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Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must deal with microscopic technic or with the nature or uses of biological stains. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

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Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of the stain should be given and the exact dye used should be specified. If this information is not known, the name of the manufacturer should be stated and that of the stain itself exactly as worded on the label. Certified stains should be specified by certification number.

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STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

BIOLOGICAL STAINS IN TIME OF WAR

The Biological Stain Commission was conceived as the result of the World War. Now it faces another European war, and it is interesting to notice the differences in the situation.

In 1914 there were no American-made dyes. All stains used in American laboratories were of German manufacture, mostly the product of two concerns, which had originated from the efforts of a single man and were often confused in this country so that the two lines of German stains were thought to be one. When the United States entered the war the various new laboratories which were set up at that time were unable to obtain German dyes and found the American products unreliable. It was to meet this situation and to put the use of stains on a more scientific basis that the Stain Commission was established.

Today, the situation could hardly be more different. Nearly every large nation has its dye industry, and that of the United States is unsurpassed. Thanks to the cooperation of the manufacturers on the one hand and numerous scientists on the other, the production of biological stains is now on a scientific basis; the manufacturers know what biologists want and the biologists know how to make any new requirements they may have intelligible to the industry. Furthermore, practically all stains and every intermediate needed for their manufacture can be obtained from American sources. There probably are *no* exceptions to this statement, altho there are a few of the less common dyes and compound stains which have been largely imported lately because of difficulties that have been obtained with some of the American products. Among these have been brilliant cresyl blue and Giemsa stain. It is interesting, by the way, to note that American stain companies report a greatly increased demand for these two particular stains since September first.

There has, as a matter of fact, been a general stimulation to the stain industry since summer. This has been reflected by the orders the stain companies have sent to the Stain Commission for certification labels. The increased demand began in August, so it could not

have been due wholly to the shutting off of the European sources of supply. September, and then again October, broke all preceding records in the demand for certification labels. This has been very gratifying, especially now that the Commission is no longer being fianaced by the chemical foundation. If the increased demand proves to be more than temporary, the Commission may become self-supporting. Users of stains can help make it permanent by taking pains to see that their institutions order Commission certified stains.

H. J. CONN

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the October number of this Journal.

STAINS CERTIFIED SEPT. 1, 1939 TO NOV. 30, 1939*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methylene blue chloride	NA-14	87%	As histological and bacteriological stain, and as constituent of blood stains	Sept. 8, 1939
Brilliant cresyl blue	NV-17	57%	As vital stain for blood	Sept. 30, 1939
Sudan IV	LZ-2	81%	As stain for fat	Oct. 13, 1939
Indigo carmine	LI-1	83%	As histological stain	Oct. 13, 1939
Fast green FCF	CGf-1	90%	As histological and cytological counterstain	Oct. 13, 1939
Methylene blue chloride	DA-4	84%	As histological and bacteriological stain, and as constituent of blood stains	Nov. 1, 1939
Basic fuchsin	NF-32	93%	For general staining, the Feulgen reaction and in bacteriological media	Nov. 2, 1939
Brilliant cresyl blue	NV-18	63%	As vital stain for blood	Nov. 8, 1939
Sudan IV	NZ-11	64%	As stain for fat	Nov. 10, 1939
Janus green B	NJ-8	73%	As vital stain for blood	Nov. 15, 1939
Crystal violet	MC-1	93%	As histological, cytological, bacteriological stain, and in bacteriological media	Nov. 21, 1939
Eosine B.	NEb-8	80%	As histological counterstain	Nov. 24, 1939
Methylene azure	LAz-1	63%	As histological stain and as constituent of blood stains	Nov. 27, 1939

Special note:—Attention is called to the two samples of brilliant cresyl blue certified within about six weeks of each other. Both were submitted by the same company. Such a short interval between samples submitted has never occurred before; it seems to indicate a suddenly increased demand for Commission Certified samples of this particular dye.

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

A STANDARDIZED TECHNIC FOR SAFRANIN O[1]

CHARLES H. SAWYER, *Yale University, New Haven, Conn.*

ABSTRACT.—A method for control of staining with Safranin O is described. The procedure is as follows:

Overstain the sections, freed of paraffin, 4 hours or more in 0.1% solutions of either light green S F or fast green F C F in 50% alcohol. These solutions are adjusted to pH 2.4 with 0.1 *N* HCl. Rinse in distilled water.

Destain at least 30 minutes in Sörensen's Buffer pH 8. Rinse in distilled water.

Overstain in 0.1% Safranin O, 4 hours or more. Rinse in distilled water.

Destain 15 minutes in 0.01 *N* HCl (pH 2) or in 0.001 *N* HCl (pH 3) depending on whether light green or fast green, respectively, is the counterstain. The acid solutions are freshly prepared from a stock solution of 0.1 *N* acid. Rinse in distilled water.

Dehydrate in two changes of dioxan, pass thru xylol and mount in balsam.

Safranin O, one of the most valuable nuclear stains, has always presented difficulties to the histologist; it has given extremely variable results (Conn, 1936, p. 86). At least a part of this inconstancy has been due to the solubility of the stain in alcohol. Destaining and dehydrating are usually performed simultaneously and often too much of the stain washes out before the preparation is mounted. The aim of the present work has been to obviate this phase of the difficulty by controlling the destaining process in an aqueous solution of definite pH and by dehydrating in dioxan (Guyer, 1936, p. 64). The author is grateful to Dr. Petrunkevitch, who devised the method (Petrunkevitch, 1937), and under whose supervision the work was done.

It was first necessary to find the optimum pH to use for destaining. A curve (Fig. 1) was prepared to enable one to make up quickly a series of 0.1% aqueous solutions of the stain from pH 2 to pH 10. The procedure was to add varying amounts of 0.1 *N* HCl or NaOH to 25 ml. samples of a stock solution of 0.2% Grübler's Safranin O and to dilute with distilled water to 50 ml. in a volumetric flask. A Beckman glass-electrode pH-meter was used in pH measurements,

[1]Contribution from the Osborn Zoological Laboratory, Yale University.

and all the readings were taken at 23° C. From the curve a stain series was set up ranging from pH 2 to pH 9.

Since staining affinity varies widely with the fixation employed, four representative fixing fluids were used: Petrunkevitch's paranitrophenol–cupric-nitrate–nitric (Guyer, p. 34), Zenker's bichromate–sublimate–sodium-sulphate–acetic (Lee, 1937, p. 46), Bouin's picro-

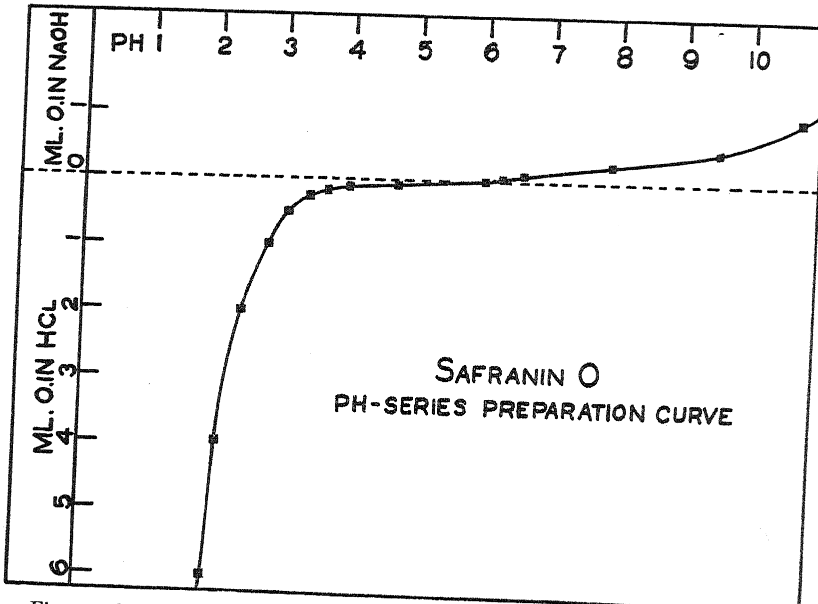

Fig. 1. Curve showing amount of 0.1 N acid or alkali needed to make up 50 ml. 0.1% Safranin O at various pH-values.

formol-acetic (Lee, p. 58), and Petrunkevitch's sublimate-nitric-acetic-alcohol (Lee, p. 45). The tissue, intestine of the leopard frog, was fixed in these fluids and sectioned at 10μ in paraffin. The sections, freed of paraffin, were stained in the pH-series overnight to insure completion of the staining reaction. Each was then rinsed in an HCl or NaOH solution of approximately the same H-ion concentration as the stain, dehydrated in two changes of dioxan, passed thru xylol, and mounted in balsam. Treatment preliminary to staining may be with either alcohol or dioxan, but the latter is quicker.

From the results (Figs. 2–5) it can be seen that the greatest differentiation between nuclear and cytoplasmic staining is in each case at pH 3 or 4 and that nearly maximal staining occurs at pH 6, the stain prepared with no addition of acid or alkali. At pH 2 the material fixed in Bouin's or Zenker's fluids shows no nucleo-cytoplasmic differentiation; maximal differentiation is reached at pH 4. The Bouin-

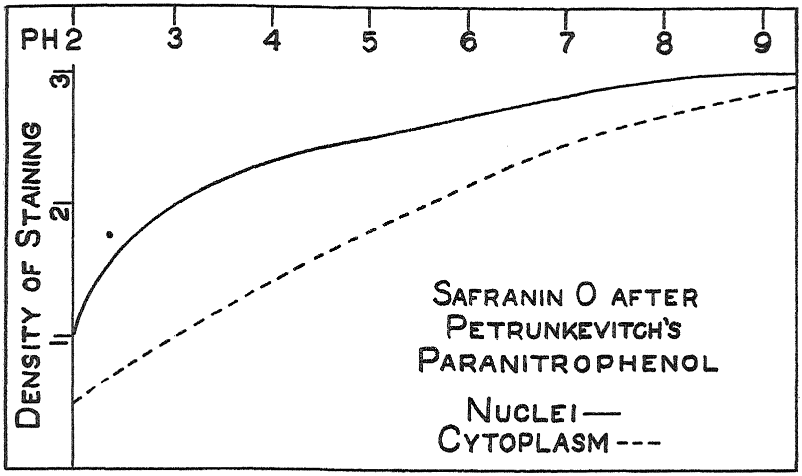

Figure 2.

fixed sections were unusual in that the secretion-filled goblet cells of the lining epithelium had a stronger affinity for the stain than did the nuclei—taking the stain even at a reaction of pH 2. The tissue fixed in Petrunkevitch's fluids showed its highest differentiation at pH 3. No color comparator was used in judging differentiation, but the results were sufficiently clear-cut so that the estimations of relative density designated on the curves as 1, 2, and 3 for slight, medium and maximum, are fairly accurate.

On the basis of these results the destaining method was attempted, keeping in mind the rule that the capacity of retaining a stain in a de-

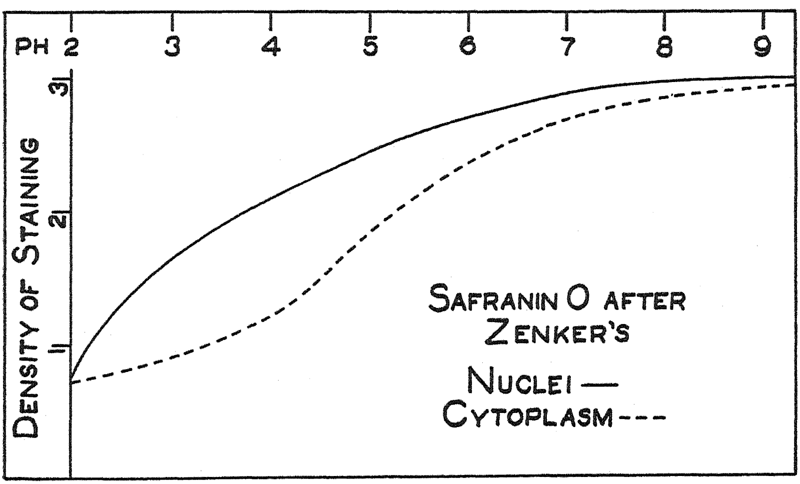

Figure 3.

staining fluid of a given pH-value is not the same as the staining affinity at that pH-value. Inasmuch as the stain without treatment by acid or alkali gave nearly maximal affinity, it was used untreated for the preliminary overstaining. Destaining at pH 2 (0.01 *N* HCl, prepared by diluting 5 ml. 0.1 *N* HCl to 50 ml. in a volumetric flask) gave the following results: Zenker-fixed material, no differentiation; Bouin-fixed, goblet cells only; Petrunkevitch's sublimate or *p*-nitrophenol-fixed, excellent differentiation, only the chromatin retaining

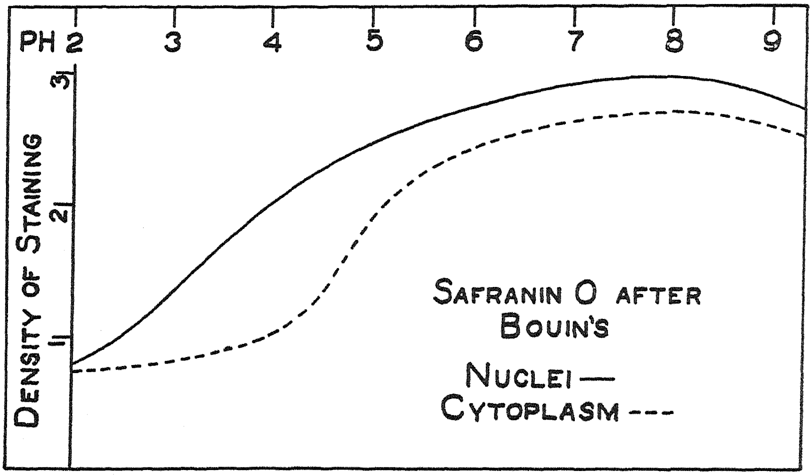

Figure 4.

the stain. Optimum destaining for Bouin- or Zenker-fixed tissue was achieved at pH 3, but the Bouin-fixed material never gave excellent differentiation. Of the Petrunkevitch fixatives the *p*-nitrophenol gave slightly the more striking contrast. Tho the destaining process is theoretically independent of the time element, provided completion of the destaining reaction is reached, prolonged exposure to pH 2 tends to make the sections slip off the slide; 15 minutes is sufficient for optimal destaining in acid.

Two green counterstains were successfully employed, light green S F (Coleman and Bell Co.) and fast green F C F (National Aniline and Chemical Co.). These gave optimum results when the sections were overstained in 0.1% solution in 50% alcohol at pH 2.4 and destained at pH 8 with Sörensen's phosphate buffer. Each of the acid staining solutions was made up by taking 10 ml. of 0.5% aqueous solution of the stain, adding 25 ml. of absolute alcohol and 4 ml. of 0.1 *N* HCl, and diluting with water to 50 ml. Best results were obtained by applying the counterstain before staining with Safranin O.

Of the two greens, light and fast, the latter is much the better from the point of view of permanency. Safranin O, however, has a weaker affinity for sections previously counterstained by fast green; so if the latter is used the final destaining is better effected at pH 3 (0.001 *N* HCl).

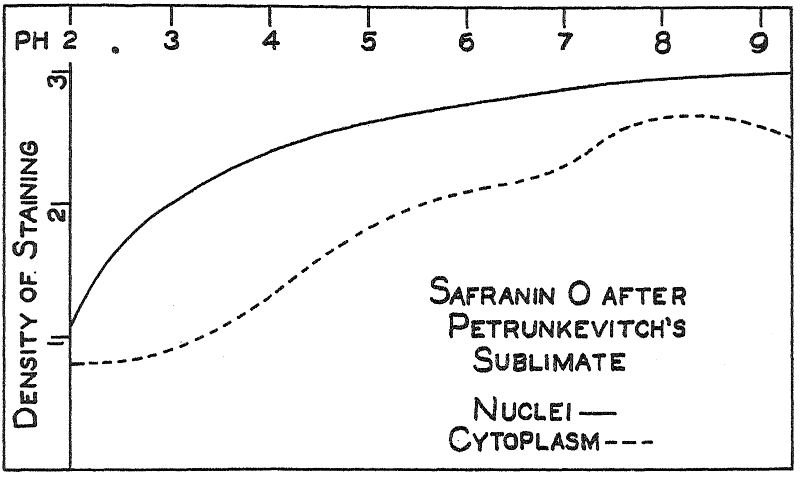

Figure 5.

Not all stains are amenable to the treatment described here. It may appropriately be reported that crystal violet gives negative results on application of this technic. One can tell only by experiment whether or not a particular stain will react favorably to the treatment. As has been stated, the procedure for Safranin O was developed using a sample of Grübler's ("water soluble"). The method has since been satisfactorily applied to a second batch of Grübler's and to a sample from the National Aniline and Chemical Co. (Certification No. NS-9, dye content 97%), so it should be generally applicable to Safranin O.

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STAINING PARAFFIN SECTIONS WITH PROTARGOL

5. CHLORAL HYDRATE MIXTURES, WITH AND WITHOUT FORMAMIDE, FOR FIXING PERIPHERAL NERVES.[1]

EDWARD W. BANK[2] and H. A. DAVENPORT, *Department of Anatomy, Northwestern University, Chicago, Ill.*

ABSTRACT.—A series of experiments was directed toward finding a means of improving fixation of mammalian glandular tissue and peripheral nerves with chloral hydrate. Specimens from cat, dog, rat, guinea pig, and man were fixed in solutions of 5–15% chloral hydrate in ethyl, methyl, and propyl alcohols, both pure and diluted with varying amounts of water. Modifiers were added, including acids, alkalies, alkaloids, amines, formamide, pyridine, piperidine, and formalin. The sectioned material was stained by the 2-hour method ($AgNO_3$-protargol) described previously (Davenport et al., 1939). The acidification of alcoholic chloral hydrate mixtures was deleterious to fixation but alkalinization was not. Among the modifiers, formamide was the one which showed definite improvement of fixation. A 10% solution of formamide alone in 50% ethyl alcohol gave good fixation and staining of peripheral nerve trunks, but addition of 5–7% chloral hydrate to this mixture improved staining. Treatment with 1% ammoniated alcohol after fixation and before embedding was of no value in section staining. Block stains were not tried.

In the preceding paper of this series (Davenport, McArthur, and Bruesch, 1939) a two-hour method for staining nervous tissue was described. The method was worked out chiefly on spinal nerve roots, tissue from the central nervous system, and sympathetic trunks. The use of acid mixtures recommended for fixing central nervous tissue and roots has not proved very satisfactory for peripheral nerve trunks and is particularly bad for glandular tissue. The present study has been directed toward improvement in fixation and differentiation of the more peripherally located nerves by the use of neutral or alkaline fixatives based on mixtures of chloral hydrate, alcohol and water. Chloral hydrate was chosen because it has been

[1]Contribution No. 303 from the Department of Anatomy, Northwestern University Medical School.

[2]Submitted by E. W. Bank as a partial requirement for the degree, Master of Science, to the Graduate School of Northwestern University.

TABLE 1. List of Fixatives*

No.	Chloral hydrate	Modifier	Alcohol	Water
1	15	0	methyl celo-solve, 100	0
2	15	0	Id. 70	30
3	15	0	Id. 30	70
4	15	0	*n*-propyl, 70	30
5	15	0	abs. ethyl, 100	0
6	15	0	" " 70	30
7	15	0	" " 30	70
8	15	0	methyl, 100	0
9	15	0	" 70	30
10	15	0	" 30	70
11	12	amm. w., 0.5‡	*n*-propyl, 60	40
12	12	quinine hydrochloride, 2	" 60	40
13	12	glacial acetic, 5	" 60	40
14	12	monochloracetic, 0.2	" 60	40
15	12	" 0.2	ethyl 60	40
16	12	0	pyridine, 50	50
17	12	amm. w., 0.25	propyl, 50 ethyl 5	45
18	12	piperidine, 0.25	propyl, 50 ethyl 5	45
19	12	*n*-butylamine, 0.25	propyl, 50 ethyl 7.5	42.5
20	12	formamide, 5	propyl, 50 ethyl, 5	45
21	12	ethanolamine, 3.5	propyl, 50 ethyl 5	45
22	12	0	propyl, 50 ethyl, 15	35
23	7	formamide, 15	propyl, 30	50
24	7	amm. w., 0.25 formamide, 15	propyl, 30 butyl, 15	50
25	0	formamide, 20	ethyl 5 butyl, 15	60
26	0	formamide, 20	py, 5	60
27	0	formamide, 20	0	80
28	0	formamide, 20 formalin, 2	0	78
29	5	brucine, 5	*n*-propyl, 50	50
30	0	" 5	" 50	50
31	5	" 5	methyl, 100	0
32**	5	Py, 20	ethyl, 40	40
33***	5	amm. w., 0.2	ethyl, 50	50
34†	3	formalin, 12	ethyl, 50 pH	50
35	7	formamide, 7	propyl, 35–4.9	50
36	7	" 7+amm. w., 1.0	" 35–8.9	50
37	7	" 7 " " 0.1	" 35–7.4	50
38	7	" 7+trichlor-acetic acid, 0.01	" 35–4.7	50

*Formamide, piperidine, *n*-butylamine, ethanolamine, and *n*-propyl alcohol were obtained from Eastman Kodak Co., Rochester, N. Y. Protargol was obtained from Winthrop Chemical Co., New York, N. Y.

**d'Ancona's fluid No. 1

***d'Ancona's fluid No. 2

†One of Cajal's formulae.

‡Abbreviations: amm. w., strong ammonia water; Py, pure pyridine (Merck).

TABLE 1. LIST OF FIXATIVES (continued)

No.	Chloral hydrate	Modifier	Alcohol		Water
39	7	formamide, 7+trichlor-acetic acid, 0.1	"	35–4.1	50
40	0	formamide, 7	"	35–4.9	50
41	7	" 7	ethyl	35–4.9	50
42	0	" 7	"	35–5.0	50
43	7	" 0	"	50	50
44	0	formamide, 10	"	50	50
45	7	" 10	"	50	50
46	7	" 10+Py, 15§	"	35	50
47	7	" 10+Py, 15+ formalin, 4	"	35	50
48	7	formamide, 10		0	100
49	7	" 10	methyl,	50	50
50	7	" 10	propyl,	50	50
51	0	0	ethyl,	50	50
52	0	0	propyl,	50	50

§Py = pure pyridine (Merck).

used successfully by previous investigators (Cajal 1907, 1910, 1929; d'Ancona 1925; and Perez 1932) for nerve endings when block staining of the Cajal type was used.

Material and Methods. Nearly all the experiments were made by using 6–10 fixatives in a group and fixing in each of these a piece of nerve and two pieces of glandular tissue, such as pancreas, thyroid, adrenal and salivary glands, from the same animal. Represented in the series were 7 cats, 4 dogs, 6 rats, 1 guinea pig, and 1 human. The tissues were fixed by immersion, and the time was about 20 hours. Embedding in paraffin, cutting, and mounting the sections on the slide were done in the routine manner. Paraffin was removed and the slides passed to water thru graded alcohol, impregnated 1 hour at 60° C. with 5% aqueous $AgNO_3$ solution, washed in 3 changes of distilled water 0.5 minutes each and put into 0.2% protargol solution at 27° C. for 1 hour. Following impregnation a rinse of 1 or 2 seconds in distilled water was given and the protargol reduced by amidol-sulfite solution (amidol, 0.1 g.; $NaHSO_3$, 1.0 g.; Na_2SO_3, 10 g.; and water, 100 cc.) for 1–2 minutes. The reducing solution was made up immediately before using by adding the dry amidol to a stock solution of the sulfites. The slides were washed carefully to remove all reducing solution and toned in a 0.1% solution of $AuCl_3$ for about 5 minutes, washed again and, if the stain were light, reduced 0.5 minutes with a 0.5% amidol solution; if dark, reduced 5 minutes with a mixture of oxalic acid and formalin (oxalic acid, 2 g.; formalin, 1 cc.; water 100 cc.). To insure the removal of unreduced AgCl, a

5–10% "hypo" solution should be used after the second reduction, but this was not done in a routine way, because the preparations were kept only a short time. A final washing followed by dehydration and covering in balsam completed the staining. The procedure has a few minor modifications of the original method.

Fixatives: The 52 fixatives listed in Table 1 are about half of the total number tried, but include those which are characteristic representatives of the various modifiers used. We had in mind the following questions:

1. Is there any advantage of methyl or *n*-propyl alcohol over ethyl?
2. What is the proper water content of these alcohols when used with chloral hydrate?
3. What is the effect of adding acid or base?
4. Does the addition of amines, amine-like substances, or alkaloids improve fixation or subsequent staining with silver?

In addition to the fixatives in Table 1, 12% chloral hydrate solutions in isopropyl, allyl, *n*-butyl, isobutyl, and octyl alcohols and in dioxan were tried. With the exception of isopropyl alcohol, excessive shrinkage occurred with their use. Our findings with regard to the higher alcohols agree with Cajal's (1907) observations on their lack of utility in similar fixing solutions for staining in the block.

In one series of tissue samples (fixatives 43–51) the specimens were cut in two after fixation and one half given a further treatment of 24 hours in 1% ammoniated alcohol. Such treatment seemed to be of no value or even deleterious to both staining and fixation, as shown by paler stains and greater shrinkage.

Methyl cellosolve and dioxan were poor substitutes for alcohol.

Results and Discussion: No advantage of methyl or propyl alcohol over ethanol appeared consistently. When used without dilution with water, propyl alcohol caused more generalized shrinkage than either methanol or ethanol. It was found that 10–15% solutions of chloral hydrate in mixtures of water and propyl alcohol separated into two layers when the water content exceeded 40%, but these could be made into perfect solutions by the addition of a small amount of ethyl alcohol, ethanolamine, formamide, ammonia or other modifiers used in fixatives 17 to 22. Ethyl and methyl alcohols gave perfect solutions when mixed with any amount of water.

The answer to question 2, regarding water content of the fixative, appears to be that only methyl alcohol can be used pure with impunity as a solvent for chloral hydrate in fixation. Ethyl and propyl alcohols cause excessive shrinkage when so used. Ethanol as 50 and 70% mixtures with water, and propanol diluted to about 40% with

sufficient formamide (fixative 35) to homogenize the solution were definitely more satisfactory than higher concentrations.

The addition of acids was deleterious, but the addition of ammonia, piperidine, pyridine, and butylamine in small amounts was not. Addition of acids, except in relatively minute amounts (fixative 38), caused serious cracking of the tissue, especially peripheral nerves, with the result that cross sections presented a mosaic-like pattern caused by longitudinal fissures—a type of artifact commonly seen after fixation in neutral formalin. In some specimens the addition of alkali favored the subsequent differentiation of nerve fibers in glands, but in others this effect was not seen. It appeared to have no effect on fixation *per se*.

Of all the modifiers tried, only one showed a consistently beneficial effect both on fixation and on subsequent staining. This was formamide, and while it was used by Cajal (1907) as a 4% solution in 96% alcohol, we have found no mention of its use in conjunction with chloral hydrate. This substance, altho it has a chemical formula like an amine, has properties more nearly resembling urea in that it is able to denature protein and is not alkaline. As an addition to chloral hydrate solutions, it seems to be well worth while. Used alone (without chloral hydrate) in water-ethanol or water-propanol mixtures (fixatives 40 and 44) it has given superior fixation and strong staining with silver. The addition of chloral hydrate to the mixture, however, appears to give some improvement in the differentiation of nerve fibers.

The use of formalin (fixatives 28, 34, and 47) was deleterious both to fixation and staining.

We wish to suggest that fixatives having formulas the same as, or similar to, those given in fixatives 23, 24, 35, 36, 41, 45, 49 and 50 are worthy of trial in the staining of peripheral nerve trunks, particularly when enumeration of fibers is desired.

Results with glandular material have been only partially successful; hence the use of chloral hydrate mixtures, altho a great improvement over acid fixatives, leaves much to be desired in section staining with the silver-nitrate-protargol method.

We wish to acknowledge aid from the National Youth Administration to E. W. Bank.

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A PROCEDURE FOR STAINING FILAMENTOUS ALGAE AND FUNGI ON THE SLIDE

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The Venetian Turpentine method for mounting delicate structures such as filamentous algae requires so much handling of the bulk material that tangling, twisting, and excessive breaking of the filaments is almost unavoidable. The conjugating filaments of forms which show scalariform conjugation are likely to be separated and otherwise distorted. Due to matting of the filaments, uniform destaining is difficult of accomplishment, and satisfactory teasing-out of the matted filaments in the thick Venetian Turpentine just prior to covering is difficult or impossible.

The following procedure, based upon the use of a modification of Haupt's[1] gelatine fixative, has been used successfully by the writer in overcoming these difficulties and in shortening the preparation time.

Dissolve 1.5 g. of a good grade of gelatin in 100 ml. of distilled water at 30–35° C. Add 5 ml. of glycerin and 2 g. of phenol. Smear a thin film of the fixative upon a warm slide.

After killing and washing the bulk material in running water remove enough for a slide with fine forceps. Place this upon the smeared slide with a "dragging" movement, beginning at one end of the slide and pulling the filaments toward the other end. The water which adheres to the filaments is sufficient for floating them into position; a large quantity of water such as is used for floating paraffin ribbons is unnecessary and undesirable. Most of the filaments will thus arrange themselves in a free and parallel fashion. Conjugating filaments can be mounted in this manner without damage. If desired, long ends extending beyond the area to be covered may be turned in beside the main mass of filaments. Invert the slide over a shallow dish containing a small quantity of commercial (40%) formaldehyde and cover with an inverted dish or low bell jar. Glass staining dishes of the low, rectangular form, slightly less than 3 inches wide, serve this purpose well. Leave the slides thus exposed to the fumes of formaldehyde for ½ hour. Wash slides in distilled water, 5–10 minutes. They are now ready for staining in aqueous stain or for the alcohol series if an alcoholic stain is to be used. Clear in xylene and mount in balsam.

[1]Haupt, A. W. 1930. A gelatine fixative for paraffin sections. Stain Techn. 5, 97–8.

The following schedule has given good preparations of *Spirogyra:*

1. Kill and fix in chromo-acetic acid (1% chromic acid, 3% glacial acetic acid), 12 hours.
2. Wash in running water, 12 hours.
3. Fix material to slide as described above. Wash in distilled water, 5–10 minutes. (Slides may at this point be run up to 85% alcohol for hardening and returned to water.)
4. Mordant in 2% aqueous solution of ferric ammonium sulfate, 4 hours. Wash in several changes of water, ½ hour.
5. Stain in 0.5% aqueous hematoxylin, 12 hours. Wash in water (2 or 3 changes), ½ hour.
6. Destain with 1% aqueous solution of ferric ammonium sulfate, about 1 hour. Wash in water, ½ hour.
7. Dehydrate by closely graded series of alcohols to 95%.
8. Counterstain with 0.5% orange G in 95% alcohol, 15–30 seconds.
9. Complete dehydration in absolute alcohol, 1–2 minutes. Clear in xylene. Mount in balsam.

Other filamentous algae with this and other combinations of stains have given good preparations. Miss Alma Whiffen of this laboratory has used successfully the present technic for mounting and staining cultures of *Achlya* grown on hemp seed.

FURTHER EXPERIMENTS WITH THE MASSON TRICHROME MODIFICATION OF MALLORY'S CONNECTIVE TISSUE STAIN[1]

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Abstract.—Several dyes, notably ponceau 2R, azofuchsin 3B, nitrazine yellow, and Biebrich scarlet may replace imported "ponceau de xylidine" in the Masson ponceau acid fuchsin mixture. Of these Biebrich scarlet appears to be the best and may be used without acid fuchsin.

A mixture of equal parts of 5% solutions of phosphomolybdic and phosphotungstic acids is much superior to either acid alone and gives adequate mordanting in 1 minute at 22° C.

With the fast green modification, times in plasma and fiber stains can be reduced to 2 minutes each. With anilin blue a 4-minute plasma stain is required. One-minute final differentiation in 1% acetic acid is adequate.

Primary mordanting of formalin material may be accomplished by 5 minutes in saturated aqueous mercuric chloride or 2 minutes in saturated alcoholic picric acid. Three minutes washing in running water is required after these mordants.

While our previous studies (Lillie, 1938) and those of Goldner (1938) indicated that the trichrome method could be adapted to formalin material and considerably shortened, the length of time (nearly an hour) and the necessity for incubation required by the best of our modifications were still a bar to the frequent or routine use of the method. Moreover, the necessity for an imported "ponceau de xylidene" for the proper execution of the method gave rise to some difficulties in prompt procurement of the dye. Consequently, we determined to test a number of other dyes as to their availability for plasma stains in place of "ponceau de xylidene," and to endeavor to further shorten the procedure.

The following basic technic was used in experiment 9.[2] Bring paraffin sections of formalin fixed tissues to water as usual. (1) Treat in mordant No. 1 (saturated aqueous picric acid) 10 minutes at 58° C. (2) Wash 15 minutes in running water. (3) Apply Wei-

[1]Contribution from Division of Pathology, National Institute of Health.

[2]Experiments 1 to 8 are included in the previous report (1).

gert's acid iron chloride hematoxylin, 6 minutes. (4) Rinse in water. (5) Stain 5 minutes in plasma stain. (6) Rinse in distilled water. (7) Apply mordant No. 2 (1% phosphomolybdic acid) 10 minutes at 58° C. (8) Stain in 2.5% fast green FCF in 2.5% acetic acid, 5 minutes. (9) Differentiate in 1% acetic acid for 2 minutes. (10) Carry thru alcohol, acetone, acetone+xylene, and xylene to salicylic balsam. The plasma stains were made by mixing 9 cc. of 1% solution of the various dyes with 1 cc. of 1% acid fuchsin and 0.1 cc. glacial acetic acid.

The best plasma stains were given by ponceau 2R, nitrazine yellow, Biebrich scarlet and azofuchsin 3B. Almost as good were Bordeaux red, chromotrope 2R, azofuchsin G, orange G and crocein. Still quite good were "ponceau de xylidene," azofuchsin (C.I. 30), azofuchsin 4G, eosin Y and chrysoidin.[3] Distinctly inferior were azofuchsin B, azofuchsin 6B (C.I. 57), azofuchsin 6B or GN (C.I. 154), methyl eosin and erythrosin. Phloxine gave the poorest effect of any. (The sources of these dyes are given in Table 1.) Appendix, pneumonic lung and a nephrosclerotic kidney were used as test objects.

Having noted the statement in Thorpe and Linstead (1933) "that the lakes of complex acids containing both tungsten and molybdenum were much faster than those containing either metal alone" (English patent 216486, 1923), it was decided to compare a mixture of phosphotungstic and phosphomolybdic acids as second mordant with the two acids used alone. In experiment 10 the usual technic was followed, employing Biebrich scarlet with acid fuchsin as the plasma stain. Second mordants were as follows: 1% phosphomolybdic acid, 10 minutes at 58° C.; 1% phosphotungstic acid, 10 minutes at 58° C.; and a mixture of equal parts of 1% solutions of phosphomolybdic and phosphotungstic acids for 5 and 10 minutes at 58° C. and for 5, 10, 15, 30 and 60 minutes at room temperature.

Phosphotungstic acid alone gave distinctly inferior results, but the mixture was excellent at 58° C. for either 5 or 10 minutes and at room temperature when the time was 10 or more minutes.

As Goldner had found he could shorten the phosphomolybdic mordanting time by using more concentrated solutions at room temperature, we decided to try in experiment 11 the effect of raising the concentration of the mixed acids to 5%. The Biebrich scarlet variant was used as before. A control of 1% phosphomolybdic and phosphotungstic acids at room temperature for 10 minutes was used,

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[3]This dye was labeled chrysoidin Y, but was shown not to agree with the characteristics of that dye.

and trials were made of a mixture of equal parts of 5% solutions of the two acids at room temperature for 1, 2, 3, 5, and 10 minutes. All tests gave fully satisfactory results.

In experiment 12 trial was made to see whether the time in the plasma stain could be reduced and whether acid fuchsin could be omitted from the Biebrich scarlet modification. Mordanting was carried on for 1 minute at room temperature with the 5% phosphomolybdotungstic mixture. Plasma stains were applied 1, 2, 3 and 5 minutes, using 1% Biebrich scarlet in 1% acetic acid alone as well as the mixture of Biebrich scarlet and acid fuchsin (9:1).

TABLE 1. DYES USED

Name	C.I. No.	Schultz No.	Manufacturer	Lot No.
"Ponceau de xylidene"			Grübler	1.37
Ponceau 2R	79		National Anilin	7895
Bordeaux red	88	112	National Anilin	4988
Chromotrope 2R	29		National Anilin	8449
Crocein	252 or 277?		Old sample	
Biebrich scarlet WS	280	247	Biosol Products	131
Azofuchsin	30		National Anilin	7788
Azofuchsin B	66		National Anilin	3210
*Azofuchsin 4G	29 a*		National Anilin	8449
Azofuchsin 6B	57		National Anilin	3210
Azofuchsin 6B, GN or S	154		National Anilin	3210
Azofuchsin G	153		Hartman Leddon	(1938)
Azofuchsin 3B	54		Hartman Leddon	(1938)
Eosin Y	768		National Anilin	Cert. NE11
Methyl eosin	769?		Grübler	Old
Erythrosin	773?		Grübler	Old
Phloxine (73%)	774		Hartman Leddon	Recent
Acid fuchsin (60%)	692	524	Hartman Leddon	Recent
Methyl blue	706		National Anilin	7624
Orange G	27		Grübler	12.37
Nitrazine yellow			(Squibbs)†	Recent
Chrysoidin (acid dye)	Not C.I. No. 20, 21, or 60		Providence Chemical	About 1930
Fast green FCF			Coleman and Bell	Recent
Anilin blue WS	707		Hartman Leddon	Recent

*This is listed in the Colour Index as similar to chromotrope 2R, C.I. No. 29.

†The lot actually used was obtained from another source and under a different name; but the company furnishing it to the writer does not put it on the market any more. A sample subsequently obtained from Squibbs labelled "nitrazine" was found to be less concentrated and had to be employed in 2% instead of 1% solution. In this paper the name "nitrazine yellow" is employed for the dye, as it was first described under that name (Wenker, 1934).

Immersion for 2 minutes in plasma stain was found fully adequate, one minute not quite enough. We considered Biebrich scarlet alone to give a better picture than the acid fuchsin mixture.

In experiment 13 trial was made to see whether the time in the

fast green fiber stain and in the acetic acid differentiation could be reduced. The timing was as follows: 2 minutes in Biebrich scarlet (alone), 1 minute at room temperature in the 5% phosphomolybdotungstic mixture and 1, 2, 3 and 5 minutes, respectively in the fiber stain with the following 1% acetic differentiation applied 1 and 2 minutes for each variant of the fiber stain. Staining for two minutes in the acetic fast green was adequate, one minute not quite adequate, and one minute differentiation in 1% acetic acid was enough.

Experiment 14 was designed to test the possibility of further reducing the mordanting time in the first mordant. Both saturated aqueous picric acid and saturated aqueous mercuric chloride, which had previously given excellent results as a mordant (Lillie, 1938), were tried at 58° C. for 2, 3, 5 and 10 minutes. The remaining steps were as in experiment 13, with 2 minutes in fast green and 1 minute in 1% acetic acid. The results were essentially identical thruout, indicating that 2 minutes at 58° C. in either of these primary mordants would be adequate. In experiment 15 the same 2 first mordants were used for 2 minutes at 58° C., and trial was made to reduce the washing interval. The balance of the procedure was as in experiment 14. Sections were washed after the first mordants for 1, 2, 3, 5, 10 or 15 minutes or briefly rinsed. A 3-minute washing was required to remove all of the visible yellow color after picric acid mordanting. No difference, however, was seen in the staining with either mordant, regardless of the length of washing.

In experiment 16 the 3-minute washing interval was adopted, the rest of the procedure remaining as before, and trial was made of saturated alcoholic picric acid (about 6%, as compared with about 1.25% for aqueous), saturated aqueous mercuric chloride and 20% alcoholic mercuric chloride as first mordants. These were tried severally for 1, 2, 3, 5 and 10 minutes at room temperature in comparison with saturated aqueous picric acid and mercuric chloride solutions for 2 minutes at 58° C. At room temperature aqueous mercuric chloride gave excellent results at 5 and 10 minutes, good at 3 minutes and irregular with poor staining of muscle at 1 and 2 minutes. Alcoholic mercuric chloride dried on the slides during the brief interval of transfer to running water and results of subsequent staining were spotty and irregular. Mordanting for 1, 3 and 10 minutes gave good results, but for 2 and 5 minutes poor, with muscle almost unstained. Saturated alcoholic picric acid gave satisfactory staining results at all mordanting durations.

In experiment 17 an attempt was made to introduce another color to render the plasma staining more differential. Instead of mixing

the dyes, it was decided to try sequence staining, with or without an intervening mordant. Two dyes were used, orange G and nitrazine yellow, a brown plasma stain, in addition to the Biebrich scarlet. 1% solutions of each were made in 1% acetic acid. Tests were made with each of these dyes, staining for 2 minutes before or after a 2-minute Biebrich scarlet stain, with or without an intervening 1-minute mordanting in 5% phosphomolybdotungstic mixture. When no intervening mordant was used, the effect obtained was the same as from Biebrich scarlet alone. When the intervening mordant was used the effect was that of the first dye alone, whether Biebrich scarlet, orange G or nitrazine yellow without trace of the color expected from the second dye. The procedure was otherwise that of experiment 16 with mordanting for 1 minute in saturated alcoholic picric acid.

Continued use showed some irregularity in plasma staining with 1 minute in the first mordant, so it seemed advisable to increase this to 2 minutes. Thus the modified procedure arrived at from the foregoing experiments is as follows:

1. Bring paraffin sections thru xylol and alcohol into saturated alcoholic picric acid for 2 minutes.
2. Wash 3 minutes in running water.
3. Stain 6 minutes in Weigert's acid iron chloride hematoxylin.
4. Rinse in water.
5. Stain 2 minutes in 1% Biebrich scarlet in 1% aqueous acetic acid.
6. Rinse in water.
7. Mordant 1 minute in a mixture of equal parts of 5% solutions of phosphomolybdic and phosphotungstic acids.
8. Stain 2 minutes in 2.5% fast green FCF in 2.5% aqueous acetic acid.
9. Differentiate 1 minute in 1% aqueous acetic acid.
10. Carry thru alcohol, acetone, acetone+xylene, and xylene (two changes) into salicylic acid balsam.

Longer staining (e.g. 3 or 4 minutes) with Biebrich scarlet is necessary when the following fiber stain is anilin blue or methyl blue.

Conclusions

1. A number of dyes may be substituted for imported "ponceau de xylidene" in Masson's ponceau acid fuchsin mixture. Among the best are ponceau 2R (C.I. No. 79), azofuchsin 3B (C.I. No. 54), nitrazine yellow, and Biebrich scarlet (C.I. No. 280). We prefer the last to any dye tried thus far. Nitrazine yellow is very good when a

brown plasma stain is desired. Biebrich scarlet makes as satisfactory a plasma stain without addition of acid fuchsin.

2. A mixture of equal parts of phosphomolybdic and phosphotungstic acid solutions is much superior, as a second mordant, to either used alone. When 5% solutions are used, the time required is 1 minute at room temperature.

3. With the fast green modification, the times in the plasma and fiber stains can be reduced to 2 minutes each. With anilin blue a 4-minute plasma stain seems indicated. One-minute differentiation in 1% acetic acid after the fiber stain appears to be adequate.

4. Primary mordanting may be reduced to 5 minutes in saturated aqueous mercuric chloride solution or to 2 minutes in a saturated alcoholic solution of picric acid, both at room temperature. Washing 3 minutes in running water after the mordant seems to be adequate.

The writer is indebted to Scientific Aide Milton Gusack for technical assistance in carrying out the experimental work herein reported.

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THE USE OF SUDAN BLACK B AS A BACTERIAL FAT STAIN

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Abstract.—Sudan black B was introduced as a specific fat stain for the detection of lipids in tissue sections by L. Lison in 1934. Saturated solutions of Sudan black B in 70% alcohol or in ethylene glycol stain the fat bodies of bacteria a deep blue-black color, and this dye is recommended as superior to the other Sudans.

The method used in staining the bacteria was to suspend a loopful of the cells in a drop of the stain solution and to prepare flat wet mounts. The organisms giving positive fat tests with Sudan black B included *Bacillus cereus*, *Bacillus mycoides*, *Azotobacter beijerinckii*, *Rhizobium leguminosarum*, *Mycobacterium avium*, *Mycobacterium leprae*, *Oospora lactis*, *Bacillus tumescens*, water spirilla, and fungi.

Introduction

The use of the Sudans as fat stains dates back to 1896, when L. Daddi introduced Sudan III as a specific stain for fat. L. Michaelis, in 1901, reported on his synthesis of Sudan IV, which had similar staining properties and was related to Sudan III. French (1926) reported oil red O, (*Syn.*, Sudan II) as the most brilliant of the Sudan fat stains.

The above mentioned Sudans have long been recognized as the stains of choice when testing cells for fat. Recently, however, Sudan black B has been proposed as having staining properties equal to the other Sudans. It was introduced by Lison (1934) as a specific fat stain for the detection of lipids in tissue sections, and is a dyestuff of the phenyl-azo-naphthyl-azo-naphthyl type. Recently, Leach (1938) proposed 50% diacetin and distilled water as an improved solvent for Sudan black B, and also presented a method of using it to stain lipids in sections of intestinal epithelium.

Since the stains of the Sudan series readily stain cell fats, an investigation was conducted to determine the ability of Sudan black B to stain the fats of bacteria and to find a more suitable solvent for the dye, other than those already proposed, which would more readily adapt itself for the staining of lipids in bacteria.

The method of staining the fats of bacteria is to suspend the organisms in the dye and prepare flat wet mounts. For this procedure

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it is of value to have a stain which will readily suspend the bacteria in question and not cause precipitates or plasmolysis of the cells due to evaporation of the dye solvent. The solvents found to meet these requirements are 70% alcohol, 50% acetone and water, 50% dioxan and water, and ethylene glycol.

Materials and Methods

The Sudan black B used during this study was furnished by the National Aniline and Chemical Company, New York City. The staining solution was prepared by dissolving 0.25 g. of the dry Sudan black B powder in 100 ml. of 70% alcohol at room temperature. Solution of the dye takes place almost immediately. The staining solution as described by Leach was prepared by adding excess of the dye to equal amounts of diacetin and distilled water followed by two days incubation at 55° C. and filtering before using.

Saturated solutions of Sudan black B in 50% acetone and distilled water, 50% dioxan and distilled water, and ethylene glycol[1] were prepared. For best results it is advisable to let the solutions stand for several days since the acetone and dioxan require considerable time for solution to take place.

The method used in staining the bacteria was to suspend a loopful of the cells in a drop of the stain solution and prepare flat wet mounts.

The organisms tested for fat during this investigation included *Bacillus cereus*, *Bacillus mycoides*, *Bacillus tumescens*, *Azotobacter beijerinckii*, *Rhizobium leguminosarum*, *Mycobacterium avium*, *Mycobacterium leprae* (strain isolated by J. R. Kriz), *Oospora lactis*, water spirilla, and fungi. (These organisms were obtained from the stock cultures of the University of Texas at Austin.)

The *Rhizobium* and *Azotobacter* cultures were grown on yeast extract mannitol agar and nitrogen-free mannitol agar respectively. The fungi studied were isolated from the air by exposing glucose agar plates for several minutes. The other test organisms were grown on 6% glycerin infusion agar and carbohydrate infusion agar.

The cultures were studied at various periods during their growth and the presence of fat bodies within them was detected by the use of Sudan black B. All positive fat tests were confirmed by the fat stains of Eisenberg (1909), by staining with Sudan II, III, and IV, by negative fat stains, and by unstained vital preparations.

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[1]The ethylene glycol used as a dye solvent should have a boiling point of 195–7° C., since if less pure it will not completely dissolve Sudan black B.

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PLATE I.

Fig. 1. *Bacillus cereus* from a 48-hour culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.

Fig. 2. *Bacillus tumescens* from a 32-hour culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.

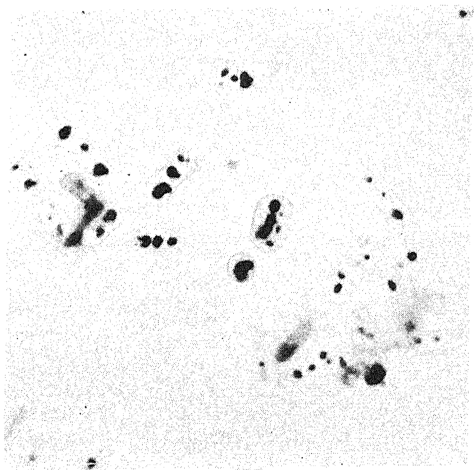

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PLATE II.

Fig. 3. *Oospora lactis* from a 48-hour culture on 6% gylcerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.

Fig. 4. Water spirillum from a 12-day culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in ethylene glycol.

Observations and Conclusions

All the bacteria used in this study gave positive results when tested for fat by the use of Sudan black B. Fat bodies could be detected in all the organisms within 24–48 hours; however, the *Mycobacteria* gave better results if tested for fat after 7–14 days incubation. The fat droplets appear as blue-black bodies in a clear colorless cytoplasm. (Figs. 1, 2, 3, and 4). At times the organisms may assume a beady or chain-like appearance, since only the stained fat bodies are visible (Figs. 1, 2, and 4) while the cell membrane is indistinct or not visible at all.

The fat bodies stain a deep blue-black color when stained with the 70% alcohol staining solution; they assume a blue color when ethylene glycol, 50% dioxan and distilled water, and 50% diacetin and distilled water are used as the dye solvents. When Sudan black B is added to 50% acetone and distilled water, a red solution is formed which stains the fat bodies of the bacteria red; this is due to the fact that many organic dyestuffs have an entirely different color in organic solvents and only give their true color when the solvent is evaporated.

The most brilliant fat tests were observed when the bacteria were stained with the dye solutions using either 70% alcohol or ethylene glycol as the solvent. Ethylene glycol seems to be especially suitable as a dye solvent for Sudan black B, since it does not tend to evaporate when making vital mounts, and in addition has the property of taking on moisture, thus permitting preparations to remain in excellent condition for several days. The other solvents gave less striking results, altho the fat bodies were stained a bright blue color. The stain solution as recommended by Leach did not give very satisfactory results, as the fat bodies stained a faint blue and at times did not stain at all. Forty per cent alcohol, which has been recommended as a solvent for Sudan black B, proved to be unsatisfactory because insufficient dye was dissolved.

Sudan black B in 70% alcohol has retained its staining ability for over six months and does not seem to have deteriorated in any way. Dye solutions using ethylene glycol, 50% dioxan and distilled water, and 50% acetone and distilled water as solvents, also have kept their staining abilities over long periods of time. Frequently a dark precipitate is observed in the wet-mount preparations; however, since it is usually in the background and beyond the focal level of the cells, it is of no objectionable consequence. Therefore, it can be concluded that the dye solvents tried in this study do not tend to deteriorate upon aging or to cause undesirable precipitates or to give poor staining reactions.

From the observations made during this study, Sudan black B is to be preferred to Sudan II, III or IV as a bacterial fat stain. The solubility of the Sudan black B in the various solvents tried, as well as the slight solubility of the other Sudans in similar solvents, is a matter of practical importance. The microscopical picture obtained when using Sudan black B as compared to the other Sudans would indicate that Sudan black B presents a less confusing picture and is equally specific for fat substances.

Summary

Sudan black B dissolved in 70% alcohol or ethylene glycol stains the fat bodies of bacteria a deep blue-black color, and is recommended as superior to the other Sudans.

The author wishes to express his appreciation to Dr. I. M. Lewis, Department of Botany and Bacteriology, University of Texas, for his helpful advice and criticism during this work.

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A SIMPLE TECHNIC FOR IN TOTO STAINING OF TARSAL AND SEBACEOUS GLANDS

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In toto or bulk staining with Sudan IV as used by Herxheimer is an efficacious method for delineating tarsal and sebaceous glands. Altho Sudan III and Sudan IV have long been used for staining fatty substances in general, the lack of references to the application of these stains for purposes referred to above indicates that the demonstration of entire tarsal and sebaceous glands is not in general use. Fatty substances in general stain more deeply with Herxheimer's stain than with Sudan III.

The eyelids and skin removed from animals (dog, ox, cat and human cadavers) were fixed in 10% formalin for 24 hours or longer and then run thru 50% alcohol (ethyl) into 70% alcohol. Care should be taken to wash out the fixing fluid thoroly with several changes of 70% alcohol. This will avoid the formation of a precipitate after the tissue is placed in the stain.

Cadavers, when received at the Indiana University anatomical laboratories, have already been injected with a preservative, the ingredients of which are unknown. The eyelids and skin removed from them for purposes designated above are fixed in 10% formalin before they are finally injected with a mixture of equal parts of alcohol, glycerin and phenol.

To delineate the tarsal glands clearly, the tissues covering the tarsus should be removed. This can be easily done by submerging the eyelid in 70% alcohol in a large Petri dish, the bottom of which has been coated with a layer of paraffin about 5 mm. thick, and by pinning the lateral and medial margins of the eyelids to the paraffin. Care should be taken not to insert the pins thru the tarsus. When the eyelid is thus anchored, the skin, orbicularis oculi muscle and the palpebral conjunctiva can be removed easily from the tarsus with a small pair of curved scissors and a pair of fine forceps. The use of binocular magnifying lenses with a long working distance is an aid in removing the excess tissue. A binocular loupe is very satisfactory to use with this type of work.

If it is also desirable to display the large sebaceous glands that open into the hair follicles of the eyelashes, the epidermal layer of the skin near the margin of the eyelids should be removed.

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For the delineation of the sebaceous glands, free-hand vertical sections 1 to 2 mm. thick are made from any region of the integument where these glands are present (scalp, skin of general body surface). The sections are more easily made from fixed than from fresh material. Whole mounts of skin 12 mm. square or larger also make interesting preparations.

The formula for Herxheimer's stain[1] is as follows:

70 parts of absolute alcohol (ethyl),
20 parts of a 10% solution of sodium hydroxide,
10 parts of distilled water,
Sudan IV to saturation.[2]

The procedure for staining is as follows:

1. Transfer tissue from 70% alcohol to Herxheimer's stain. Staining is accomplished in 12–24 hours or in less time if the pieces of tissue are thin.

2. Wash out excess stain with repeated changes of 70% alcohol until the glands are sharply delineated.

3. Transfer to glycerin. The tissues surrounding the glands become semitransparent. The preparation may be kept permanently in glycerin, or may be mounted in glycerin jelly. Of the several formulae for glycerin jelly, Brandt's formula[3] was used: melted gelatin 1 part; glycerin 1½ parts; and a few drops of carbolic acid.

Tarsal and sebaceous glands stained by this method seven years ago have not faded and make beautiful whole mount preparations to supplement the study of these glands in ordinary microscopic preparations.

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[1]Enzyklopädie der Mikroskopischen Technik, Dritte Auflage, Bd. 1, 729–30. Urban und Schwarzenberg, Berlin-Wien.

[2]The dye used for staining the majority of the specimens was labeled "Scharlach R" and purchased a good many years ago from the Harmer Laboratories of Lansdowne, Pa. For some of the recent specimens a lot marked "Scarlet R" obtained from the Coleman and Bell Co. was employed. It was subsequently pointed out by the Chairman of the Biological Stain Commission that altho the name "Scarlet R" denotes an entirely different type of dye to the dye industry it is often incorrectly employed in the medical literature as a synonym of Sudan IV. He accordingly furnished a sample of a dye sold under the latter name by the National Aniline and Chemical Co. and certified by the Stain Commission; this sample was tried and gave equally good results.

[3]Lee, Bolles. 1937. The Microtomist's Vade-mecum, 10th ed., edited by J. B. Gatenby and T. S. Painter. Churchill, London. (See p. 223).

LABORATORY HINTS

FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi *Abstract Editor*

BOOK REVIEWS

BECKER, ELERY R. and ROUDABUSH, ROBERT L. **Brief Directions in Histological Technique.** 6 x 8½ in., 80 pp. Paper, with spiral binding. Collegiate Press, Inc., Ames, Iowa. 1939. $1.00.

According to the authors' statement in the preface, "This manual was prepared especially for use by classes in histological technique in the Zoology and Entomology Department at Iowa State College." Altho the book makes no pretention of having general applicability, it may prove useful in some other laboratories where an inexpensive handbook of this sort is desired. The writers give detailed directions for one standard histological procedure (Zenker-paraffin with Delafield's hematoxylin), and follow this with alternate procedures for the various steps involved. The chapter dealing with the standard procedure is about 18 pages long; and this is followed by five shorter chapters dealing with the alternate procedures for fixation, dehydrating, imbedding, staining, and mounting, respectively. A final chapter deals with special methods for tissue or microörganisms to which the more general histological technic is not applicable.—*H. J. Conn.*

MICROSCOPE AND OTHER APPARATUS

MELEZER, N., and VENKEI-WLASSICS, T. **Die Quecksilberhochdrucklampe als Lichtquelle für Fluoreszenzmikroskopie und Mikrophotographie.** *Zts. wiss. Mikr.*, **56**, 202–10. 1939.

The author suggests the use of a new tungsten-mercury-vapor lamp developed by the Simons Company in place of the expensive low voltage ultraviolet quartz lamps or the unsatisfactory ultraviolet arc lamp using iron electrodes. These lamps, designed on the general plan of the ordinary tungsten incandescent lamp with Edison base, burn evenly emitting a constant source of light having a wave length between 310–390 mμ. They operate on 220 volts A. C.—*J. M. Thuringer.*

SCOTT, G., and PACKER, D. M. **The localization of minerals in animal tissues by the electron microscope.** *Science*, **89**, 227–8. 1939.

Partial absorption of an electron beam is known to give rise to profile pictures of bacteria, or epidermal cells. Such a beam can also be used to cause thermoionic excitation of electrons on metallic surfaces. This principle is applied here in an attempt to localize minerals in sections of animal tissues. Sections are ashed at greatly reduced pressure on the surface of a cathode coated with Ba and Sr in the electron microscope. As most of the inorganic elements in tissues are excited to thermoionic emission at relatively specific tempertaures, a differentiation and localization of at least Mg and Ca is thus made possible. A picture of cellular structures in striated muscle, gastric mucous membrane, and nerve tissue can then be obtained.—*J. A. de Tomasi.*

ZELLER, A. **Ein neues Kanadabalsamfläschchen.** *Zts. wiss. Mikr.*, **56**, 211. 1939.

A simple and practical balsam bottle is described having a large ground glass cover. An ingenious perforated groove around the neck of the bottle, beneath the cover, permits a return flow of any balsam spilled.—*J. M. Thuringer.*

MICROTECHNIC IN GENERAL

LANDAU, E. **Appareil permittant la déshydration et l'enrobage d'une pièce dans le vide, sans recourir aux substances chimiques.** *Bull. d'Histol. Appl.*, **16**, 13–8. 1939.

The author's apparatus for fixing fresh tissues by drying while freezing is described. With this apparatus, fresh, unfixed tissue can be dehydrated without employing any of the usual chemical reagents and with no shrinkage of the tissue. Dehydration is accomplished by a system of three pumps giving an almost complete vacuum, while a gas refrigerating system lowers the temperature to about -25°C. During this process the tissue may rest upon paraffin which can then be melted by means of a warm water bath. The tissue (still in the vacuum) is thus automatically immersed in paraffin. Infiltration and embedding are completed in 15–20 min.—*M. Noble Bates.*

MILOVIDOV, P. **Die Anwendung der Azetokarmin-Methode für die Färbung von fixierten Mikrotomschnitten.** *Zts. wiss. Mikr.*, **56**, 67–9. 1939.

The technic recommended has been employed for material fixed in each of the following fluids: Flemming, Němec IIIa, Nawaschin, Němec-Milovidov, Němec I, sublimate-acetic, alcohol, *etc.* Deparaffinized sections are covered with a few drops of Schneider's aceto-carmine and gently heated for 1–2 min. (without boiling). After cooling, they are rinsed in water and transferred thru 96% alcohol, abs. alcohol, xylene, to Canada Balsam. Best cytoplasmic staining is obtained after alc. or sublimate-acetic fixation. The best general results are obtained with Flemming's, Němec's IIIa, and Nawaschin's fluid. This method has the advantages of rapidity and permanency.—*J. M. Thuringer.*

ROBINSON, B. G. **A note on mounting thin celloidin-sections.** *J. Roy. Micro. Soc.*, **59**, 79. 1939.

Some of the difficulties encountered when mounting thin sections of material embedded in celloidin and paraffin wax can be eliminated by the following method. Infiltrate the material in the usual way. Then immerse the trimmed block in abs. alcohol until perfectly transparent; harden in xylene and transfer to the waxes. Float ribbons of sectioned material on slides previously smeared with albumen, stretch them on the hot-plate, drain and immediately store in a closed glass vessel containing cotton-wool soaked in ether and a small dish of $CaCl_2$. The ether vapor flattens the sections and the $CaCl_2$ drys them. Sections may be stored indefinitely in such a vessel. Before the ether, alcohol and staining baths, a photographic squeegee-roller may be lightly rolled over the slides; the slides are then able to withstand immersion in watery reagents for several days without the loss of a single section from the series.—*H. E. Finley.*

SUTER, HANS. **Über die Eignung der Schlierenmethode zur Messung osmotischer Zustandsgrössen.** *Protoplasma*, **31**, (3), 421–53. 1938.

The author presents in detail the application to biological material of the Töpler-Schlieren method (Töpler, A., Ann. d. Phys., 4, 838. 1904) which depends upon the diffusion of substances of different refractive indices in a solution. A leaf of *Sempervivum tectorum* is carefully removed and the cut base of the leaf sealed with paraffin after the epidermis has been carefully stripped off. The leaf is then placed in water or in a sugar solution, and by appropriate illumination, striae or the so-called Töpler-Schlieren can be detected in accordance with diffusion gradients and density changes in the medium over the exposed cell sap.—*Robert Chambers.*

WOLF, JAN. **Über die Herstellung mikroskopischen Präparate der Obefläche verschiedener Objecte mit Hilfe der Adhäsionsmethode.** *Zts. wiss. Mikr.* **56**, 181–201. 1939.

The author describes the production of microscopic preparations of various surfaces with the aid of the "adhesion" method. This interesting new process utilizes celloidin casts of surfaces which may be examined by transmitted light because the images develop at the point of contact of air with the differently refracting celloidin medium. To obtain a cast of the surface epithelium of the

epidermis, the following procedure is used: (1) Paint the dry surface with a thin coat of celloidin and allow to dry from ½ to 1 min. (2) Place a piece of transparent cellophane tape over this area, avoiding the formation of air bubbles between the two layers. (3) Remove tape and celloidin with a quick movement. (4) Place on coverglass (adhesive surface down) and mount on glass slide. It may be noted that it is the under or inner surface of the cast that becomes visible on examination. The results are very interesting and instructive. The method permits modifications, *i.e.*, surface cells may be removed *in toto* and examined against the celloidin matrix or removed with adhesive tape alone and transferred to slides for examination in transmitted light with or without staining.

The method promises to become useful in histology, zoology, botany, pharmacognosy, and criminology.—*J. M. Thuringer.*

DYES AND THEIR BIOLOGICAL USES

COLLANDER, R., and VIRTANEN, E. **Die Undurchlässigkeit pflanzlicher Protoplasten für Sulfosäurefarbstoffe.** *Protoplasma*, **31** (4), 499–507. 1938.

The authors champion the findings of Plowe (Protoplasma, **12**, 196. 1931) and of Chambers and Kerr (J. Cell. Comp. Physiol., **2**, 105. 1932) that sulfonic acid dyes, altho highly diffusable, do not penetrate living cells. The present authors used varying concentrations of light green, acid fuchsin, and orange G in aq. media buffered with citrate and phosphate to pH 1.9 to 8.5. The material they used was the mycelium of *Aspergillus niger* which had been claimed by Bünning (Flora N. S., **31**, 87. 1936) to take up these dyes. They also found that orange G and cyanol are not taken up by *Tolypellopsis stelligera* even from concentrated solutions.—*Robert Chambers.*

HÖBER, R., and BRISCOE, P. M. **Correlation between secretion of dyestuffs by the kidney and molecular structure of these dyes.** *Proc. Soc. Exp. Biol. & Med.*, **41**, 624–6. 1939.

Some 30 mono-azo-sulfonic acid dyes were tested by perfusing isolated frogs' hearts with Ringer's solution and then with 0.0005% dye solutions. The position of the sulfonate group appeared to be the controlling factor in secretion or failure to secrete the dye. The suggestion is offered that, if sulfonate groups are attached to only one-half of the molecules, the sulfonated half may be hydrophilic and the other half may be hydrophobic and organophilic, permitting anchoring between the cell and surroundings as the first step in penetration.—*M. S. Marshall.*

KEMPTON, R. T. **Differences in the elimination of neutral red and phenol red by the frog kidney.** *J. Cellular and Comp. Physiol.*, **14**, 73. 1939.

The excretion of phenol red by the normal and narcotized frog kidney has been studied in relation to changes in pH of the urine. It was found that in contrast to that of neutral red, the phenol red elimination was unaffected by changes in urinary pH. As with neutral red, phenol red elimination was stopped in the narcotized kidney. This inhibition of phenol red elimination is explained by an interference with cell activity, whereas that of neutral red is apparently caused by the alkalinizing of the urine subsequent to the stopping of acid excretion.—*L. Farber.*

KRAJIAN, ARAM A. **A new frozen section method for the preparation of permanent frozen sections of loose texture tissues.** *Amer. J. Clin. Path., Tech. Suppl.*, **3**, 189–92. 1939.

Add 2 cc. of formalin to 50 cc. of fresh uncontaminated pooled human or animal serum. This keeps several weeks at room temp. Fix loose texture tissues in 10% formalin 24 hr. or longer. Trim and wash to remove formalin. Put in serum and keep in a warm place (e.g., top of paraffin oven) over night. Cover any floating sections with absorbent cotton. Decant excess of serum and add dioxan. Stand 3–5 hr. or until completely coagulated. Freeze, cut, stain and mount as usual. Preserve blocks in 10% formalin; dioxan causes shrinkage. For emergency, put thin blocks of formalin fixed tissue in serum in a paraffin oven at 56° C. 1–2 hr. Coagulate with dioxan 1 hr.—*George H. Chapman.*

YOE, JOHN H., and BOYD, GEORGE R. **Patent blue V as a pH and redox indicator.** *Ind. Eng. Chem., Anal. Ed.*, 11, 492–3. 1939.

An aq. solution of patent blue V may be used as an indicator for the colorimetric determination of pH over the interval 0.8 to 3.0. The colors range from yellow thru green to blue and are stable for periods up to 5 days, after which a very slight fading may be detected. The change in color is pronounced and the pH of a solution within the range given above, can be determined to 0.1 of a pH-unit by comparison with standards made up at intervals of 0.2 pH. The same dye sample must be used for both standards and unknowns because solutions of the dye from different sources often exhibit slightly different colors at a particular pH-value.

Patent blue V can also be used as an oxidation-reduction indicator in certain volumetric methods. Altho it cannot be used with $K_2Cr_2O_7$ or in the presence of HCl, it can be used with $KMnO_4$ or $CeSO_4$ if all HCl has been removed. The oxidation-reduction potential of the purified indicator was 0.78 volt. This value corresponds almost exactly to the potential at the equivalence point in the ferrous-ferric system—namely, 0.77 volt.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

BAYLEY, J. H. **Differential staining methods for formalin-fixed human pituitary gland.** *J. Path. & Bact.*, 49, 261–3. 1939.

The technic described for differential staining of the granule cells in the anterior lobe of the pituitary has the advantages of being applicable after ordinary formol fixation, of permitting good staining of basophil granules, and of eliminating mitochondrial staining. The following are considered the most useful and permanent methods.

General procedure: Fix whole gland in 10% neutral formol-saline for 12 hr., bisect and replace in formol for a further 24 hr. Prepare paraffin sections as usual; xylene, 2 min.; abs. alcohol, 1 min.; sat. aq. $HgCl_2$, 2 min. (to improve later staining); Lugol's iodine, 2 min. To avoid staining mitochondria, bring sections to dist. water; 0.5% $KMnO_4$, 1 min.; 5% oxalic acid, 2 min.; rinse well with tap-water. Stain 2 min. with hot but not boiling 1% aq. acid fuchsin (Grübler). Rinse in dist. water. Stain 1–3 min. in the following solution: Acid violet (Revector, supplied by Vector, Ltd.), 1 g.; orange G, 0.3 g.; dist. water, 200 cc. Rinse in dist. water. If overstained in the acid violet mixture, differentiate with 80% alcohol or in extreme cases with Lugol's iodine. Rinse; dehydrate in abs. alcohol, 2 min.; xylene, 2 min.; mount in balsam. Eosinophils are crimson, basophils dark blue, chromophobe cells grey, erythrocytes crimson, and connective tissue grey.

For connective tissue proceed as before until staining. Stain 5 min. in hot but not boiling 1% aq. acid fuchsin; rinse; stain 1–3 min. in the following: acid violet (Revector), 1 g.; orange G, 0.5 g.; acid green (Revector), 1 g.; dist. water, 200 cc. Rinse; differentiate with Lugol's iodine (5 sec.-1 min.); abs. alcohol, 2 min.; xylene, 2 min.; Canada balsam. Muscle is dark red, fibrous tissue dark blue.—*S. H. Hutner.*

CARTER, W. **The use of prontosil as a vital dye for insects and plants.** *Science*, 90, 394. 1939.

Corn seedlings, with or without roots, take up neoprontosil (a red dye) very rapidly. Their leaves show red streaks within even a few seconds, and insects, like the corn leafhopper, will show presence of the dye in their tissues after feeding on dye-saturated leaves for a day or two.—*J. A. de Tomasi.*

DONAGGIO, A. **Dimostrazione dell' esistenza di una lesione organica reversible nell' azione degli anestetici sulle fibre nervose centrali e periferiche.** *Arch. exp. Zellforschung*, 22, 171–80. 1939.

The following method is said to leave only the anesthetized nervous tissue stained: Fix 36 hr. in Zenker's fluid; rinse in dist. water; place in amber colored aq. iodine (a few drops of tincture of iodine in dist. water) for 48 hr.; rinse in dist. water; place in 2.5% $K_2Cr_2O_7$ 4–5 min. at 37 C.; dehydrate in a series of alcohols; embed in celloidin; section at 20μ; immerse sections in Lugol's solution (5g. KI,

0.5g. I_2, 5g. water, 45g. 90% alcohol) to eliminate traces of ppt.; wash in 95% alcohol for 1 hr.; pass thru 70% and 50% alcohol to dist. water, a few min. in each; stain in stannic hematoxylin (add 1% aq. hematoxylin at least a week old to an equal amount of 20% aq. $SnCl_4$) for six hr.; partially decolorize by Pal's method (0.1% $KMnO_4$, followed by a mixture of 1 g. oxalic acid, and 1 g. K_2SO_3, or preferably $Na_2S_2O_3$, in 200 cc. dist. water); examine under low power until the color is removed from the normal tissue.—*V. W. Kavanagh.*

GOMORI, G. **The effect of certain factors on the results of silver impregnation for reticulum fibers.** *Amer. J. Path.*, 15, 493–5. 1939.

The impregnation of reticulum fibers by ammoniated alkaline silver reagent (Gomori, Amer. J. Path., **13**, 993–1002. 1937) is only slightly affected by fixation. The following fixing fluids were used with organs from man, dog and guinea pig: alcohol, Carnoy's fluid, formalin-alcohol (1:5), formalin (1:10, presumably aq., but not stated), Bouin's, Orth's, Zenker's, Stieve's, and Zenker-formalin (9:1). Carnoy's gave deep black staining of reticulum fibers with gray nuclei and almost unstained cytoplasm. Cytoplasm, but not nuclei, were stained after Bouin's; other fixatives gave results similar to formalin. Exhaustive oxidation of the sections by 2 or 3 treatments with acid $KMnO_4$ (0.5% H_2SO_4 added to 0.5–1% $KMnO_4$) alternated with decolorization by a 1–3% solution of $K_2S_2O_5$ intensified the differentiation between reticulum fibers and other tissue elements. Length of time of fixation in formalin from 12 hr. to several months did not affect staining, neither did decalcification with either nitric or sulfosalicylic acid. Thin sections, 8μ or less, are preferred.—*H. A. Davenport.*

JACOBSON, W. **The argentaffine cells and pernicious anemia.** *J. Path. & Bact.*, 49, 1–19. 1939.

The granules of the argentaffine cells of the gastro-intestinal tract may be stained by taking advantage of their ability to reduce silver salts and to couple with diazotizing agents. For both methods tissue may be fixed in 10% formol-saline or 10% neutral formol, dehydrated with alcohol, cleared in cedarwood oil or in methyl benzoate plus 2% celloidin, and imbedded in paraffin.

The following modified Masson-Hamperl silver staining technic was used: Wash deparaffinized sections in glass-dist. water (10 min. is enough if two changes of water are used); place 12–24 hr. in Fontana's solution (prepare by adding NH_4OH to 5% $AgNO_3$ until the precipitate is dissolved, then add more $AgNO_3$ drop by drop until the fluid shows a slight persistent opalescence; glass-dist. water must be used); wash slides 1 min. in glass-dist. water; 5% $Na_2S_2O_3$, 1 min.; tapwater, 10 min. Only the granules of the argentaffine cells appear black. Nuclei may be counterstained with carmalum and the sections permanently mounted in Canada balsam.

An alternative quicker diazo method is as follows: prepare diazotizing solution by dissolving small amount of *p*-nitro-methoxybenzene diazotate (Kernechtrotsalz B) in dist. water to produce a light yellow solution. Alkalinize with a small amount Li_2CO_3. About ½ min. later pH 10–11 is reached, the color changing to dark orange-yellow. Take sections from dist. water; immerse in stain, 30–40 sec.; wash 1 min. in dist. water. Granules of argentaffine cells are dark red against a yellow background. Nuclei can be counterstained with hemalum.—*S. H. Hutner.*

KATÔ, HIDEHARU. **Über den Einfluss der Fixierung auf das Hirngewicht.** *Folia Anat. Jap.* 17, 237–97. 1939.

This study on the influence of fixation on brain weight was based on the examination of an extensive series of mouse, rat, cat, dog, monkey, and human brains fixed respectively, in Müller's, Helly's, Orth's, Zenker's, Ciaccio's, Carnoy's fluids, in alcohol and in 10% or 20% formalin.

It was found that Müller's, Helly's, Zenker's fluids and formalin produced an increase in weight while Carnoy and watery alc. solutions caused the brains to lose weight. Ciaccio's fluid did not influence the weight appreciably one way or another. Formalin fixation of human brains produced an average increase in weight of 10%–12% in 3–4 days, whereupon the weight remained constant for about 3 months, then diminished slowly to an average of 1.9% above their original weight after 24 months fixation.

The author stressed that brains of various animals reacted in a definite individual manner. The results are given in 37 tables and 7 graphs.—*J. M. Thuringer.*

LEPLAT, G. **Des avantages de la glycérine dans la déshydration des tissus conjunctifs et des os, avant l'enchâssement.** *Bull. d'Histol. Appl.*, **16**, 118–21. 1939.

Impregnation with glycerin is recommended to counteract the hardening and shrinking effect of the higher alcohols upon tissues which previously have been decalcified.

Good sections of the acoustic epithelium with Reissner's membrane always intact are obtained when (after decalcification with an aq. solution of HNO_3, treatment with Na_2SO_4, and washing) the labyrinth is placed for 2–3 days in each of the following: (1) a solution of 1 part glycerin to 3 parts dist. water, (2) a solution of equal parts glycerin and water, (3) pure glycerin. Transfer for 2 hr. to abs. alc. to complete dehydration and to remove the glycerin. Place in an abs.-alc.-ether mixture. Embed in celloidin.

Difficulty in obtaining serial sections of bone dehydrated in the usual manner is overcome if glycerin is employed in the process. Pieces of bone decalcified, washed, and run up to 50% alcohol are treated as follows: (1) 50% alcohol, 6–10 hr. (2) Anhyd. glycerin and 70% alcohol in equal parts, 24 hr. (3) Pure glycerin, two changes in 24 hr. (4) Abs. alc., 2 hr. (5) Clear in cedar oil and embed in paraffin.

In other tissues which are difficult to section serially (but which do not contain bone), the author finds that a certain quantity of glycerin added to the higher alcohols (70%–95%) in the dehydration process results in easier and more regular sectioning.—*M. Noble Bates.*

PERDRAU, J. R. **Ammonium molybdate as a mordant for Mann's stain and the Weigert-Pal method.** *J. Path. & Bact.*, **48**, 609–10. 1939.

For consistent Mann staining, treat material fixed in mercuric mixtures as follows. Place sections, Hg-free, in 2% ammonium molybdate overnight at 37° C. Wash thoroly in dist. water for 5–10 min.; leave in Mann's mixture overnight. Wash in dist. water and differentiate in 70% alcohol tinged with a trace of orange G, controlling the process under the low power of the microscope. Dehydrate, clear and mount. Overmordanting and overstaining is impossible.

With Mann's original staining solution the following results are obtained: Chromatin, nuclear membrane, cell membrane and connective tissue are blue; nucleoli, erythrocytes, cytoplasmic inclusions and many cell granules bright red; the rest pale pink. With other fixatives the background may be blue instead of pink.

Old bichromate-hardened frozen sections of nervous tissue that have been kept a long time in alcohol after hardening can be made to stain properly, myelin included, by mordanting in ammonium molybdate.—*S. H. Hutner.*

POURSINES, Y. **Techniques de coloration myélinique et cellulaire (type Nissl) du tissu nerveux, sur coupes à la paraffine provenant d'un même bloc.** *Bull. d'Histol. Appl.*, **16**, 128–34. 1939.

The steps of a myelin technic for paraffin sections are described. A 4–6 day ferric impregnation of the tissue by a 5% aq. solution of ammoniacal iron alum is done before embedding. (To secure a good impregnation, the formalin-fixed tissue has to be treated first with 95% alcohol followed by an ether-alcohol mixture).

Mounted sections are mordanted in 5% iron alum solution, stained with Regaud's hematoxylin, and differentiated with $KMnO_4$ and 22% iron alum. Myelin sheaths are stained dark gray or black.

Other or alternate sections cut from the same block may be stained to show Nissl bodies, or may be stained with hematoxylin and eosin, by first treating them with oxygenated water or with HCl, so as to modify the iron compounds deposited in the tissue during the ferric impregnation. Directions are given for this treatment and the staining with Unna's polychrome blue.—*M. Noble Bates.*

SCHROEDER, KURT. **Eine weitere Verbesserung meiner Markscheidenfärbemethode am Gefrierschnitt.** *Zts. gesam. Neurol. u. Psychiat.*, **166**, 588–93. 1939.

For staining myelin sheaths in frozen sections, the following technic is recommended:

Fix nervous tissue in formalin as usual. Cut 20–30 μ frozen sections. Mordant 24 hr. at 37° C. in a mixture 1 vol. of Müller's fluid and 2 vol. of Weigert's rapid mordant. (Composition of the rapid mordant is not given, but presumably consists of CrF_3 2.5 g., water 100 cc., heated to boiling in a covered vessel, with 5 cc. glacial acetic acid and 5 g. of copper acetate added in the order given). Wash quickly in dist. water and transfer to the staining solution made as follows. Add 3 cc. of any kind of 10% alc. hematoxylin solution to 100 cc. dist. water and boil 5 min. Cool and add 3 cc. sat. aq. soln. $LiCO_3$. Make up to 100 cc. if there is much loss in boiling. Stain sections for 12 hr. or longer at 37° C. Wash well and treat with 0.25% aq. $KMnO_4$ about ½ min. Wash twice in dist. water during the next minute and differentiate in a solution made by mixing equal parts of 1% aq. oxalic acid and 1% aq. K_2SO_3. Keep the sections in motion during differentiation and change the solution after ¼ to ½ min. Continue the differentiation until the gray matter is clear, then soak 15 min. in sat. aq. $LiCO_3$, 1 cc., plus tap water, 100 cc. Wash thoroly, dehydrate and mount in balsam. The method can be used for gelatin embedded frozen sections with the following modifications: Mordant 5 days at 37° in Müller's fluid, stain several days, use 1% $KMnO_4$, and double the concentration of the differentiator.—*H. A. Davenport.*

SPEK, J. **Studien über die Polarität der Larven der Kalkschwämme.** *Protoplasma*, **30** (3), 352–72. 1938.

The author used traces of dil. solutions in sea water of brilliant vital red (Grübler), Nile blue sulfate B, and brilliant cresyl violet on the blastulae of *Sycandra setosa* and *Leucandra aspersa*. Particularly with brilliant cresyl violet, he obtained striking color differences (from blue to red) in the different cells of the blastulae. These color differences he ascribed to differences in pH of the cytoplasm. His illustrations indicate that the color appears in the granular contents of the cells.—*Robert Chambers.*

SPOERRI, ROSETTE. **A new material for mounting nerve tissue sections in paraffin for silver staining or restaining.** *Science*, **90**, 260. 1939.

Egg albumin is not always a satisfactory fixative for nerve tissue sections. The following starch paste was found reliable: suspend 1 g. starch in 10 cc. cold water, add 20 cc. boiling water, and stir until homogeneous. Add 2 drops HCl, and boil 2–5 min. After cooling, preserve with a small crystal of thymol. Use like albumin, allowing the sections to dry on the slide 3 days at 45° C.

For Ag impregnations of sections on slides, the technic is as follows: fix material in formalin, section in paraffin, and affix to slides. Dry; pass thru xylene into water, and up thru graded alcohols to pyridine for 15 min. Put in fresh pyridine overnight. Wash 10 min. in dist. water, and impregnate 3 hr. in the dark in 5% $AgNO_3$. Transfer for 30 min. to ammoniacal $AgNO_3$ (5% $AgNO_3$, 200 cc.; 10% NaOH, 5 cc.; with NH_4OH added drop by drop until precipitate dissolves). Wash rapidly in dist. water, soak 5 min. in 10% formalin. Wash; tone in gold, or fix 2 min. in "hypo"; dehydrate; clear; and mount.—*J. A. de Tomasi.*

TERRY, R. J. **A thoracic window for observation of the lung in a living animal.** *Science*, **90**, 43–4. 1939.

An instrument has been devised suitable for observation of the superficial air sacs and alveoli in the cat's lung. It consists of a bronze cylinder with a small coverglass window mounted at one end, and provided at the other with a quadrilateral plate or flange. The instrument is placed in position thru an opening in one of the intercostal spaces in the thoracic wall, so that the end-plate rests against the inner surface of the wall while the window faces the lung. When the air in the pleural cavity is taken out by exhaustion, the lung surface comes to rest against the window and can be studied most conveniently. Observations are carried out with a binocular and a skin microscope. A motion picture of local phenomena has been obtained.—*J. A. de Tomasi.*

WALLART, J. **Essais de coloration de l'hypophyse.** *Bull. d'Histol. Appl.*, **16**, 149–52. 1939.

In an attempt to distinguish more clearly between the different cells of the hypophysis, the author developed a modification of existing staining methods. After preparing the sections in the usual way, they are stained as follows: Cover with Masson's ponceau-fuchsin or 1% acid fuchsin, 5 min.; wash briefly in dist. water; add 5% phosphotungstic acid, 5–8 min.; drain the surplus without washing; add Krall's anilin blue or Hollborn's standardized anilin blue, 3–5 min. with slight agitation; rinse quickly in running water; immerse in 1% acetic acid in dist. water, 30 min.; 1% acetic acid in abs. alcohol, ½–1 min.; abs. alcohol; toluene; Canada balsam.

The results are: Chromatin of the nuclei ordinarily blue, sometimes reddish or even bright red, especially in degenerating nuclei; the nucleolus most often bright red but sometimes bluish; karyoplasm sometimes colorless, sometimes bluish to deep blue; the acidophiles red; the basophiles blue; granules of the principal cells gray-blue; erythrocytes red to blue-gray.—*Jean E. Conn.*

PLANT MICROTECHNIC

BALDWIN, J. T. **Chromosomes from leaves.** *Science*, **90**, 240. 1939.

The following smear method for chromosomes from leaves is a modification of Warmke's technic (Stain Techn. **10**, 101–3, 1935): Fix young leaves in Carnoy's for 5 min. or longer; pass briefly thru 95% alcohol and conc. HCl, 1:1; carry back into Carnoy's, and after a few min. smear out in the aceto-carmin.—*J. A. de Tomasi.*

MICROÖRGANISMS

BROADHURST, JEAN and PALEY, CHARLES. **A single-dip stain for the direct examination of milk.** *J. Amer. Vet. Med. Assoc.*, **94**, 525–6. 1939.

This staining procedure is essentially that employed in the Newman technic for the direct microscopic count of bacteria in milk. It differs in the inclusion of basic fuchsin for the staining of the background material. This, in the opinion of the authors, makes for more accurate counts with less eye strain.

Preparation of Stain. Add 0.4 cc. of concentrated H_2SO_4 (measured accurately) to 54 cc. of 95% alcohol. Mix with 40 cc. of technical tetrachlorethane in a flask and heat to about 55° C. (no higher). Add the combined solution while hot to from 1.0 to 1.2 g. of methylene blue; shake until dye is dissolved. Add 8.0 cc. of a 1% solution of basic fuchsin in 95% alcohol. Mix well, cool, filter, and store in tightly stoppered bottles.

Directions for Use. Prepare milk smear as usual, by spreading 0.01 cc. of milk on a slide over an area of 1 or 2 sq. cm., as preferred. Dry the smear on a flat surface in a warm place within 5 min. When dry, dip the slide in the stain or flood the slide with the stain for about 15 sec. Drain off excess stain and dry while flat in a warm place. Wash in cold water until all the blue is washed out of the smear and it assumes a faint pink color. Dry and examine under an oil immersion objective.—*A. Zeissig.*

DELAPORTE, B. **Sur les acides nucléiques des levures et leur localisation.** *Rev. Gen. de Botanique*, **51**, 449–82. 1939.

Zymonucleic acid was extracted in relatively large quantities from living yeast cells, without affecting the nuclei at all, by washing them 2 hr. with pure water or 0.1–0.2% $NaHCO_3$ solution. With this washing, however, metachromatin granules (volutin) disappeared from the cytoplasm of *Saccharomyces ellipsoideus* and were much reduced in other yeasts. When the cells were killed with acetone, all the zymonucleic acid was extracted from the cells with the water or acetone, but still the nuclei were intact and gave positive Feulgen reactions. Thymonucleic acid extraction technics removed the material giving the Feulgen reaction from the nuclei and concentrated it in the solutions, analysis of which showed that the nuclear nucleic acid of yeasts was either thymonucleic acid or an unidentified acid very closely related to it, derived from a glucoside containing thymine, and reduced in the 2-position. The nucleus was left intact even after the second extraction and the nucleolus still stained with ferric hematoxylin.

A solution of methylene blue or cresyl blue (concentration not given), added for a few seconds either to living yeast or to smears that had been fixed in alcohol for less than ½ hr. or by flaming, stained the small metachromatin granules in the vacuoles red or reddish violet in contrast to a pale blue cytoplasm. To complete the test Meyer's reaction was used by treating the slide stained with methylene blue with 1% H_2SO_4 for a few seconds; this removes the color except from the metachromatin granules which appear black.—*V. W. Kavanagh.*

GELEI, GÁBER VON. **Neue Silbermethoden im Dienste der Protistenforschung. (Komplexsilberverbindungen).** *Zts. wiss. Mikr.*, **56**, 148–80. 1939.

The author has modified the methods of Achucarro by introducing other fixatives, reducing agents, and complex organic silver combinations. He has also substituted a sublimate-potassium-dichromate-alum mixture ($HgCl_2$, 7%; $K_2Cr_2O_7$, 2.5%; $AlK(SO_4)_2 \cdot 12H_2O$, 1%) for the usual formalin-sublimate mixture and developed a special tannin-silver technic which gives certain results in the impregnation of cilia and the basal apparatus.

A) Tannin-silver method: (Modification consists in using tannin as a mordant.) (1) Sensitize the living material in $FeCl_2$ solution prepared by adding 1 drop of 0.1% $FeCl_2$ solution to each ml. of culture fluid. The addition of 2 drops of 1% OsO_3 to every ml. of fixed solution improves the stain. (2) Fix in sublimate-dichromate-alum, 1 min. (3) Wash in dist. water, 2 changes. (4) Cold sat. soln. tannin (Merck) heated to 50° C., 2 min. (5) Wash in dist. water. (6) Place in 1% ammoniacal $AgNO_3$, ½ min. (7) Rinse in several changes of dist. water. (8) Dehydrate in alcohol series. (9) Pass thru xylene to balsam. The method gives good results with various elements of the ciliates but not with the conduction apparatus.

B) By replacing tannin in the above technic with pyrogallol-carbonic-acid (2-3-4 trihydroxy benzoic acid), three new morphological elements were stained; namely, the elastic infra-ciliary reticulum, longitudinal fibrils of the excretory apparatus, and the sheath of the neuroneme.

C) Further modifications of the ammoniacal $AgNO_3$ basic method: (1) Essential requirement: a very active *Paramecium* culture at least one week old prepared either with a boiled straw-hay infusion or fresh horse manure (1 piece to 3 liters tap water). (2) Fix in sublimate-dichromate-alum solution, 1 min. (3) Wash in 2 changes of dist. water. (4) Conc. aq. soln. pyrogallol-carbonic-acid, 2–15 min. (cold sat. soln. plus a very slight excess crystals). (5) Wash in dist. water. (6) Ammoniacal $AgNO_3$ (Rio-Hortega), 1 min. (7) Wash in 2 changes dist. water. (8) Pass thru 40% alcohol, and glycerin-alcohol; mount in glycerin alcohol. The excretory organs were demonstrated with specially good results by the introduction of other organic silver preparations such as ammoniacal silver acetate, citrate, oxalate, lactate, benzoate, salicylate, and carbonate in place of Bielschowsky's ammoniacal $AgNO_3$.—*J. M. Thuringer.*

NIETHAMMER, ANNELIESE. **Mikroskopische Bodenpilze als Begleiter in Früchten und Samen.** *Arch. Mikrob.*, **10**, 13–25. 1939.

In the course of an investigation as to the presence of fungi in seeds and small fruit, the author has developed the following technic:

Wash the fruit or seed thoroly with sterile dist. water. Section quickly, transfer the tissue pieces to a sterile slide and cover quickly with a flamed cover glass. Introduce sterile solution of Oxaminblau (further identification and strength not given) at the edge of the cover glass and allow to stain. Transfer tissue to a suitable sterile nutrient medium and allow to remain several hours. The stain carried over is not detrimental and a distinct growth of the mycelium takes place. This stain gives a good differentiation of tissue.—*Merritt N. Pope.*

STONE, W. S. and REYNOLDS, F. H. K. **A practical method of obtaining bacteria-free cultures of *Trichomonas hominis*.** *Science*, **90**, 91–2. 1939.

The isolation of the protozoon *Trichomonas hominis* is carried out in a 16 in. capillary tube drawn from an 8 in. length of 6 mm. pyrex tubing, plugged with cotton and sterilized by dry heat. The capillary is bent into a series of small traps, in the manner of a gas analysis tube. The method follows: by means of suction, fill the tube with a sterile culture liquid (Ringer's, 1 vol.; horse serum, 8 vol.); flame and seal the distal end of the capillary, and after a 48 hr. sterility

test, inoculate the medium. Incubate at 37° C., keeping the tube vertical. The protozoa will slowly migrate toward the bottom of the tube where they can be observed under the microscope, free from bacteria. Cut off and seal the capillary in a flame, drop for 1 hr. in tincture of iodine, drain off excess, and cut capillary segments into selected culture media.—*J. A. de Tomasi.*

WALTON, SETH T. **A quick and reliable method for staining Gonococcus smears.** *J. Lab. & Clin. Med.*, **24**, 1308–9. 1939.

The author gives a modification of the Pappenheim-Saathof stain. The modified solution is prepared as follows: Methyl green (dye content 60%), 1.0 g.; pyronine B certified, 0.2 g.; abs. methyl alcohol, 10 cc.; 2% aq. phenol, 100 cc.; glycerine C.P., 20 cc. Dissolve dyes by intermittent shaking for 2 days, employing a mechanical shaker. Fix films in the usual way, add dye to warm slides and let stand for 20–50 sec. The dye is selective for the *Neisseria* group.—*Sara A. Scudder.*

HISTOCHEMISTRY

ARENS, K. **Lokaler Nachweiss von Kalzium in den Membranen des Elodeablattes mittels Natriumoleat.** *Protoplasma*, **31**, (4), 508–17. 1938.

Elodea leaves remain alive for 24 hours in 0.5% solution of sodium oleate. The precipitate which gradually forms on the surfaces of the cell walls is claimed to be calcium oleate since the chemical reaction of the precipitate corresponds to that of calcium oleate.—*Robert Chambers.*

MALLORY, FRANK B. and PARKER, FREDERICK, JR. **Fixing and staining methods for lead and copper in tissues.** *Amer. J. Path.*, **15**, 517–22. 1939.

To demonstrate the presence of lead, tissues must be fixed in 95% or abs. alcohol. Formalin is worthless. The procedure recommended is: Stain celloidin sections at 54° C. for 2–3 hr., rarely longer, in the following solution: 5 to 10 mg. (not more) of hematoxylin dissolved in a few drops of abs. or 95% alcohol and mixed with 10 cc. of a freshly filtered 2% aq. solution of K_2HPO_4. After staining, wash in several changes of tap water for 10 min. to 1 hr.; dehydrate in 95% alcohol; clear in terpineol; and mount in terpineol balsam. Lead is stained a light to dark grayish blue, and nuclei a deep blue. Another method consists of staining 10–20 min. in a 0.1% solution of methylene blue in 20% alcohol. (Sources of dyes are not stated.) Differentiate 10–20 min. in 95% alcohol. The methylene blue stain works on Zenker-fixed paraffin sections and is particularly effective when phloxine is followed by methylene blue. Copper can be demonstrated after either formalin (neutral aq.) or alcohol fixation. The same hematoxylin stain is used as for lead. Hemosiderin (Fe-pigment) stains black while copper hemofuscin stains blue. The methylene blue stain colors the Cu-pigment pale blue but does not color the Fe-pigment. The probable specificity of the staining reactions was checked on tissues of animals acutely poisoned by Pb and Cu salts.—*H. A. Davenport.*

MILOVIDOV, P. **Bibliographie der Nucleal- und Plasmalreaktion.** *Protoplasma*, **31** (2), 246–66. 1938.

Almost twenty pages of references are listed on the subject of Feulgen's specific staining reaction of chromatin. Among these are included those dealing with the aldehyde reaction which may also occur in the cytoplasm.—*Robert Chambers.*

TONUTTI, E. **Ergebnisse histochemischer Vitamin C-Untersuchungen.** *Protoplasma*, **31** (1), 151–8. 1938.

The author reviews the technic of Giroud and Leblond (*Arch. Sciences et Ind.*, **435**, 1936, Paris) for cytological demonstration of vitamin C as follows: Short washing of specimen in isotonic levulose solution (5.4%); transfer to 10% $AgNO_3$ containing two drops glacial acetic per cc. (up to ½ hr.); rinse in dist. water for ¼–½ hr., changing the water; transfer to 3% $Na_2S_2O_3$, ¼–½ hr.; rinse in dist. water, ¼–½ hr.; transfer to 70% alcohol for paraffin imbedding. These steps should be performed in a dark-room with red light. Counterstain with "Kernechtrot" and light green. Sections should be thoroly dehydrated and protected from light.—*Robert Chambers.*

STAIN TECHNOLOGY

VOLUME 15 APRIL, 1940 NUMBER 2

THE PRESENT SITUATION CONCERNING GIEMSA STAIN

Giemsa stain is well known to be a mixture of eosinates of the oxidation products of methylene blue. The basis of this stain is the product developed by Giemsa under the name of azure I. The method of manufacture of this product was turned over by Dr. Giemsa to the firm Hollborn & Sons which is the successor of one of the laboratories founded by Dr. Grübler. The exact method of preparation has never been divulged, but the product is known to be an oxidation product of methylene blue, presumably a mixture of two or more chemical compounds. MacNeal[1] indicated this product to be a mixture of two compounds which are now designated azure A and azure B. Of these, MacNeal regarded azure B as having little if any staining value.

From azure I, Giemsa prepared another product, which he called azure II, by adding to it equal parts of untreated methylene blue. An eosinate prepared from this he denoted "azure-II-eosin" and this eosinate is at least the main constituent of the dry Giemsa stain. The liquid Giemsa stain is a solution of this, with additional azure II, prepared by a formula published by Giemsa.

Following MacNeal's suggestion that azure B is of no staining value, recent American efforts to obtain an equivalent of the Giemsa products have called for the use of azure A prepared by the method of Holmes and French[2] and presumably free from azure B. A few American stain companies have for several years been producing Giemsa stain, with azure A its main constituent, and have been submitting the products thus prepared to the Stain Commission for certification. Most of these products have been approved because they have given excellent results when tested by the technic that has been employed by the Commission. In granting this approval, however, chief stress has been laid upon performance as a stain for fixed thin films of blood.

[1]MacNeal, W. J. 1925. Methylene violet and methylene azure A and B. J. Inf. Dis. **36,** 538–46.

[2]Holmes, W. C. and French, R. W. 1926. The oxidation products of methylene blue. Stain Techn., **1,** 17–26.

During the past decade, however, Giemsa stain has come into wide use for staining unfixed thick blood films for the diagnosis of malaria, particularly in surveys of the incidence of blood infection, conducted by state, county, and city health departments. Since this development has been accompanied by increasing complaints as to the behavior of American Giemsa stains, the question has arisen as to whether the stain hitherto approved by the Stain Commission is suitable for this thick film technic. Investigations of this point have been carried on with the coöperation of the U. S. Public Health Service, and indicate very clearly that only a few of the American samples are satisfactory for the technic in question.

The thick film method of malaria diagnosis was based on a technic introduced by Ross but which has been modified by others and was finally standardized in 1929 by the U. S. Public Health Service for use in malaria survey work. In this method, thick films are made by spreading 3 to 5 drops of blood over a circle of about 15 mm. diameter on a scrupulously clean slide. These films are thoroly air-dried in a horizontal position and protected from dust and insects; this ordinarily requires 18 to 24 hours at room temperature. The slides are then allowed to stand on edge for 45 minutes in a Giemsa solution diluted in neutral distilled water or with buffer solution. The latest technic recommended by the Public Health Service calls for a dilution of 1 to 50 in distilled water buffered with phosphates to a pH-level of 7.0 to 7.2. After staining, the smears are washed 5 to 10 minutes in neutral distilled water, or preferably water buffered to pH 7.0 to 7.2, in order to clear the background. By this technic the hemoglobin should be laked out of the red corpuscles and the malarial parasites should show with clear red chromatin and clear blue cytoplasm. A Giemsa stain which does not show this picture is regarded as unsatisfactory.

The laboratories of the Public Health Service report the most consistently satisfactory results with German Giemsa stain. They have used this widely for the purpose and have recommended it to other laboratories. They have tried samples of Giemsa stain prepared by various American manufacturers from time to time; but generally speaking these have not given *consistently* satisfactory results. Recently, however, on account of the impending scarcity of imported Giemsa stain, an acute demand has developed for an American product satisfactory for staining malaria parasites by the thick film technic described above.

The Biological Stain Commission has offered to help in improving this situation and has agreed to adopt the thick film technic as a

criterion of a satisfactory Giemsa stain just as soon as it is possible to give manufacturers some idea as to how to produce a stain satisfactory for this purpose. In the meantime, the coöperation of certain workers in the U. S. Public Health Service and in some of the State Health Laboratories has been made available; and all samples of this stain recently submitted to the Commission have been tested by one or more of those taking part in this coöperation.

In this way it has been found that of seven recently certified samples, three proved satisfactory by this thick film technic; namely LGe-1, GGe-5, and NGe-5. All of these were certified in 1939. GGe-5 is available in solution form, the other two in dry form. Investigations are in progress to indicate why these particular samples are superior to the others and how to standardize the manufacture of the stains so that consistently good results can always be expected. Until this is done, however, the wisest thing seems to be to urge those who use this method to purchase, when possible, one of the above-mentioned three batches of certified Giemsa stain.

Accordingly, within the last few months, the U. S. Public Health Service has sent a circular letter to state health officers in 13 of the southern states where malaria is prevalent. In this letter they call attention to the handicap arising from the present shortage of German Giemsa stain and mention the coöperation that is being undertaken with the Biological Stain Commission to determine which of the present certified products are most satisfactory. In view of the approaching malaria season (the letter continues) it seems well to get the information to the health officers at an early date. Accordingly, the certification numbers of the three satisfactory batches above mentioned are given together with the names of the manufacturers.

The Stain Commission also stands ready to furnish the names of manufacturers of these batches to anyone interested in getting the information.

This is a very empirical method of standardizing Giemsa stain. It is hoped that a more scientific method of standardization can be accomplished later. In the meantime, this statement should be of assistance to those anxious at the present time to secure a lot of stain satisfactory for the method under discussion.—H. J. CONN.

CARD MOUNTS FOR HANDLING ROOT TIPS IN THE PARAFFIN METHOD[1]

L. F. Randolph, *Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Department of Botany, N. Y. State College of Agriculture, Cornell University, Ithaca, N. Y.*

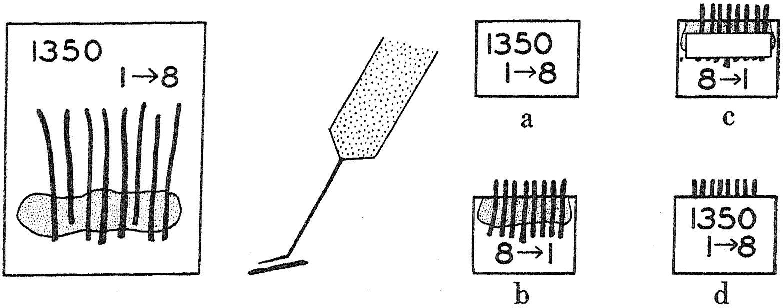
Abstract.—**A method is described for utilizing card mounts to facilitate the handling of root tip samples and similar material in the paraffin method. The freshly collected roots are attached to temporary card mounts having dimensions of approximately 2 cm. × 2.5 cm. with DuPont Household Cement or a similar adhesive that hardens rapidly in the ordinary aqueous fixing fluids and is insoluble in the lower grades of alcohol. After fixation and dehydration to 75% alcohol, the roots are transferred to permanent card mounts on which they are carefully oriented for sectioning. Mucilage or glue which hardens rapidly in 85% alcohol and is insoluble in the ordinary dehydrating and infiltrating media is used in making the permanent card mounts. Detailed instructions are given for preparing and handling the card mounts, and a system of labeling the mounts is also suggested.**

The necessity of handling separately numerous samples of root tips and similar material in the various steps of the paraffin method may be obviated by mounting the roots on cards which can be handled collectively in a single container. These card mounts also provide a means of orienting the roots in groups for sectioning so that a considerable number may be mounted on a single slide in an orderly arrangement without losing the identity of individual roots. Altho developed primarily for preparing root tips for chromosome counts, the technic is applicable to other types of material and may also be used to good advantage in comparing fixing, dehydrating and staining reactions.

The essential features of the method include the making of temporary card mounts (Fig. 1) at the time of fixation, followed after fixation and partial dehydration by a transfer of the roots to permanent card mounts on which they are carefully oriented for sectioning (Fig. 3). If desired the temporary mounts may be stored in-

[1]Coöperative investigations of the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Department of Botany, N. Y. State College of Agriculture, Cornell University, Ithaca, N. Y.

definitely in 70% alcohol. When relatively few samples are to be taken or when many roots of the same kind are to be fixed, the temporary mounts may be dispensed with, the roots being fixed in vials in the usual manner and subsequently arranged on cards in an orderly manner for dehydrating, embedding and sectioning. For preparing



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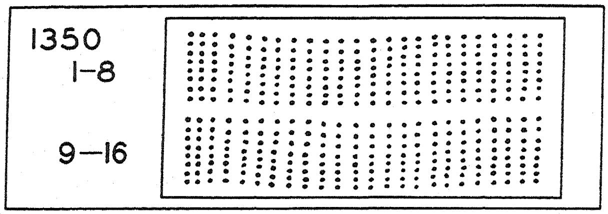

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Figs. 1–4. Diagrams illustrating the preparation of card mounts and prepared slides of root tips in the paraffin method. *Fig. 1.* Temporary card mount of fresh root tips attached to the card with household cement. *Fig. 2.* Root tips detached from the temporary card mount are transferred individually to the permanent mount with a bent dissecting needle. *Fig. 3.* Steps in the preparation of permanent card mounts: *a)* One side of the card labeled to identify the individual roots; *b)* Roots with tips projecting 2–3 mm. attached to the other side of the card with glue or mucilage; *c)* A narrow strip of stiff paper is placed over the roots to secure them more firmly in position for sectioning; *d)* The finished permanent card mount as it appears from the labeled side. *Fig. 4.* Prepared microscope slide on which was mounted a ribbon from each of two card mounts containing cross sections of sixteen root tips.

the temporary mounts an adhesive is used that hardens rapidly in ordinary aqueous fixing fluids and is insoluble in the lower grades of alcohol. The permanent mounts are prepared with an adhesive that hardens rapidly in 85% alcohol and is insoluble in the higher alcohols and paraffin infiltrating media.

The following directions may be helpful in preparing and handling the card mounts.

1. Prepare temporary card mounts from small pieces of heavy paper (heavy weight filing cards are suitable stock material) with dimensions of approximately 2 cm. × 2.5 cm. Smear the base of the card with DuPont Household Cement or some similar adhesive that hardens rapidly in the ordinary aqueous fixing fluids. Place the freshly collected roots in position on the card and add more cement, leaving at least 0.5 cm. of the tips of the roots free (Fig. 1). Invert the card with attached roots at once in the fixing fluid, keeping the cards separated for a few moments until the cement has partially hardened. This can be accomplished more readily if a shallow container is used for the fixing fluid.

2. Prepare permanent card mounts from somewhat smaller pieces of heavy paper with dimensions of approximately 12 mm. × 15 mm. After the roots attached to the temporary mounts have been fixed and transferred to 75% alcohol, the tips of the roots are removed from the card in a petri dish containing a small amount of 75% alcohol. The card on which the roots are to be mounted permanently is first labeled on one side (Fig. 3a), and then coated on the other side with a thin layer of mucilage or glue. For this purpose the clear, amber-colored grade of Carter's or Stafford's glue, evaporated to the consistency of heavy syrup, is most satisfactory; it hardens rapidly in 85% alcohol and is soluble in the reagents ordinarily used for infiltration with paraffin wax. The root tips are then transferred rapidly, one by one, from the petri dish to blotting paper for removal of the excess alcohol, and then to the card, care being taken to orient the roots approximately as desired for sectioning transversely (Fig. 3b). Add more mucilage and place over the roots a thin strip of paper as shown (Fig. 3c). Immerse the card with attached roots at once in a petri dish containing 85% alcohol. For transferring the roots quickly from the blotting paper to the card, a bent dissecting needle applied to the moist surface of the root is very effective (Fig. 2). The final orientation of the roots on the card may be completed immediately after the transfer to 85% alcohol before the mucilage hardens. The root tips should project 2–3 mm. beyond the edge of the card and must be kept free of mucilage since it interferes with the sectioning of the roots. Very small roots have a tendency to become dried out and shriveled during the mounting process; this difficulty may be lessened by placing the card on a moist filter paper during the mounting procedure and by mounting the roots, if possible, in a cool moist atmosphere free of air currents.

3. After the mucilage has hardened, which ordinarily requires but a few minutes, the mounts are placed together in a small pyrex glass beaker and dehydrated and infiltrated in the usual manner. A revised and much abbreviated schedule[2] is used in this laboratory for handling the root tip mounts in the paraffin method. The mounts should be embedded with the labeled side down so that they can be identified readily in the paraffin blocks, and if they are placed in the microtome in this same position, the ribbons can be placed on the slide with the roots in an orderly sequence (Fig. 4).

With a little practice and careful adherence to the instructions given above, the average technician should have little difficulty in preparing satisfactory card mounts. The use of these mounts in our experience saves time and reduces the amount of alcohol and other reagents required for handling large numbers of roots. Their use also eliminates errors that may result from handling numerous samples individually. In addition, it is advantageous to be able to section a number of roots at one time and to mount a considerable number in an orderly manner on a single slide. This is an especially valuable feature when comparing fixing and staining reactions.

[2]Randolph, L. F. A new fixing fluid and a revised schedule for the paraffin method in plant cytology. Stain Techn., 10, 95–6. 1935.

THE USE OF ACENAPHTHENE IN POLLEN TUBE TECHNIC

CARL P. SWANSON, *Biological Laboratories, Harvard Univ., Cambridge, Mass.*

ABSTRACT.—A procedure is described for growing pollen tubes in such a manner that a large number of clearly analyzed figures can be obtained. The pollen grains are sown on an artificial medium of sugar, agar, gelatin, and water, the proportions of each varying with the species of pollen grain used. The medium is smeared on the slide while still hot to insure a thin covering, and the pollen grains are dusted on when the medium has sufficiently cooled and hardened. The slides are placed in a staining dish provided with slide slots and a cover, the inside of the cover and the bottom of the dish being lined with moist, but not wet, filter paper. Acenapthene crystals are lightly sprinkled on the bottom of the dish. The developing pollen tubes are thus exposed to the fumes given off by these crystals with consequent disturbance to the spindle mechanism. As a result, the chromosomes are not crowded on a metaphase plate but are widely separated in the tube facilitating any observations to be made.

The present technic was developed in order to facilitate an analysis of a large number of chromosomes in the pollen tubes of *Tradescantia* L. Previous technics were found inadequate because of the small number of suitable figures at any one time, and because the nature of the work demanded a considerable number of observations in order to arrive at a statistically significant analysis. Generally the chromosomes in passing down the tube are clumped in such a manner as to make observations difficult, if not impossible. A method was therefore sought whereby this objection might be remedied.

The method of growing the pollen tubes was essentially that described previously by Newcomer,[1] the medium consisting of 2 g. cane sugar, 0.5 g. agar, and 0.5 g. gelatin in 25 cc. of water. The sugar, agar and water were brought to a boil, and then as the medium cooled somewhat the gelatin was added slowly, stirring all the while to prevent clumping of the gelatin. The medium was smeared on the slides while still hot, two slides being prepared at a time. This procedure allows the first slide to cool sufficiently to permit sowing of the grains by the time the second is prepared. If the flowers are

[1]Newcomber, Earl H. 1938. A procedure for growing, staining, and making permanent slides of pollen tubes. Stain Techn. **13**, 89–91.

plentiful, a whole one per slide may be used by either dusting the pollen on or lightly brushing the opened anthers across the slide. If the number of flowers is limited, single anthers may be used. Clumping of the grains in any one spot results in poor orientation of the tubes, lessening the number of observations which might be made. Pollen grains buried in the medium do not grow as well as those resting on the surface. After sowing the grains, the slides are placed in a horizontal type staining dish. These dishes are provided with slots so that by placing the slides back to back it is possible to prepare 20 slides at once. The writer has taken precautions not to let the slides dry out while others were in the process of preparation, but in some instances the medium can become quite stiff and still give good germination. The staining dish was previously prepared by placing moist filter paper on the under side of the cover and on the bottom of the dish. It is very important that the paper be *moist, but not wet,* for

Fig. 1. A prophase in the pollen tube of *Tradescantia.*

excess water within the dish accumulates on the medium in droplets, causing reduced germination and poor development of the growing tubes. The staining dish may finally be placed in a moist chamber at room temperature as an additional precaution against drying out.

In order to obtain a large number of suitable figures it is desirable to stop the division of the generative nucleus at a time when the chromosomes are sufficiently contracted to permit an analysis, i.e., at metaphase. Colchicine was tried, with some success, but did not satisfy the demands of the experiment. Acenaphthene (naphthylene-ethylene) [$C_{10}H_6(CH_2)_2$] was suggested, and proved to be very satisfactory. Its action is similar to that of colchicine insofar as nuclear reaction is concerned. The spindle mechanism is definitely disturbed, the chromosomes merely dividing in place without moving to the poles. The solubility of acenaphthene in water is apparently very slight—chemical handbooks give no definite figures. With the crystals scattered on the bottom of the dish, its effectiveness appears to be dependent upon the penetration of the pollen tubes by the sub-

stance thru the medium of fumes. No attempt has been made to incorporate acenaphthene into the medium.[2]

Another advantage may be cited which makes the use of acenaphthene desirable. Usually the generative nucleus passes into the tube while in an early prophase stage, the nuclear membrane being still intact. Occasionally, in untreated material, the nucleus is retained in the pollen grain until a later stage, and when the chromosomes pass down, they do so in a sort of Indian-file fashion. Altho our knowledge of the action of acenaphthene on the nucleus is still in an experimental stage, there is definitely a greater number of tubes showing this peculiar chromosome arrangement in the treated material than in the untreated. The advantage of this phenomenon to clear observation and concise analysis of the individual chromosomes is obvious. Some slides will show over one hundred good figures.

The length of time needed for development of the nucleus to its metaphase stage limits somewhat the use of acenaphthene. Experi-

Fig. 2. The arrangement of the chromosomes in the pollen tube when subjected to the acenaphthene treatment.

ments with *Bellevalia* Lapeyr. proved its ineffectiveness to act in a short period of time, this genus requiring only 3½ to 4 hours for the gametic division to be completed. *Tradescantia*, which requires 17–22 hours for development in artificial culture, responds very satisfactorily. Dahl (unpub.) has had similar good success with *Anemone* pollen which passes thru division about 38 hours after the time of sowing. Treated material is generally somewhat slower in development than that which is untreated.

Fixation of the pollen tubes in acetic alcohol (70 cc. of 100% ethyl alcohol to 30 cc. glacial acetic acid) gave beautiful preparations in some instances, but it was not always dependable. Belling's acetocarmine was used in staining. This method is by far the easiest and most rapid, and with firm pressure applied on the cover slip, a goodly number of figures can be obtained with all chromosomes on the same focal plane. For permanent slides, any of the standard fixatives and

[2]The acenaphthene used in this work is listed as 597, chemically pure, M.P. 93–94°, by the Research Laboratory, Eastman Kodak Company, Rochester, New York.

stains may be used. The use of orange G as a counterstain is undesirable since it is retained by the medium.

Growing pollen tubes in this manner likewise provides a convenient method of determining chromosome counts and chromosome morphology where other material such as root-tips and flower buds are not available. In some instances these pollen tube preparations are to be recommended over sectioned material and smears. Relational coiling can also be observed in the tubes to good advantage. This method implies, of course, using only pollen grains which can be grown on artificial media, and which have the gametic divisions in the tube and not in the grain.

POST-MORTEM AUTODIGESTION OF THE INTESTINAL MUCOSA OF THE TURKEY

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ABSTRACT.—**A gradient of post-mortem activity exists in the small intestine of the turkey. It is manifested by immediate disturbance of intestinal epithelium of the duodenum, a somewhat similar but lessened effect in the jejunum, and a long delayed action in the ileum. The recognition of such a gradient stresses the rapidity with which one must proceed in handling such tissues for technical studies. In preparing intestinal tissues for histological examination, the duodenum and jejunum must be removed immediately after killing the specimen, opened, washed carefully with warm saline to eliminate excess debris, ferments and mucous, and fixed forthwith.**

During the course of some histological work on the small intestine of the turkey, the author repeatedly obtained abnormal pictures of the mucosa from apparently normal birds. The material had been fixed by several methods: (1) dropping a small sector directly into the fixative, (2) opening and pinning before immersion in fixative and (3) filling a short length of intestinal tube with fixative and tying it off in sausage fashion. The interval before the fixative was actually applied to the tissue varied from 5–45 minutes after the bird had been killed. Such delay was necessary because samples of several tissues were being taken from each bird and some time was required to complete the manipulation. The duodenum in every case, upon sectioning, showed what appeared to be an immerse amount of sloughing of the epithelium on the tips of the villi. This regular result was found less often in the jejunum and rarely in the ileum. Besides the sloughing of the epithelium at the tips, one frequently found that the epithelium of the body of the villus was separated from the membrana propria upon which it rested. This separation often continued into the crypts of Lieberkühn.

The foregoing conditions appeared so regularly that it was first considered to be "normal". One might assume that the rough type of chyme leaving the gizzard would tear off the villus tips. Rapid replacement of that epithelium would then ensue from the proliferating cells in the crypts. The presence of denuded villus cores with the consequent possibility of infections of various types seemed, how-

ever, to preclude this condition as a normal phenomenon in the living bird. In consequence, a faster and more refined method of fixation was tried.

Immediately after killing a bird, the abdomen was opened and a portion of duodenum removed, pinned open on a piece of cork, washed in normal saline at 39° C. and inverted in 10% neutral formol at the same temperature, the whole operation being completed within 5 minutes after the bird's head had been chopped off.

A second sample of duodenum was set aside, unopened, for 30 minutes before placing it, still unopened, in the warmed formol.

Samples of jejunum were fixed similarly within 10 minutes and after 30 minutes post mortem.

The accompanying photographs show the comparative results of rapid and delayed fixation on the four samples. Sectors opened, washed and fixed at once (Figs. 1, 2) show smooth contours on the villus plates, with the epithelium intact, while material from both

TABLE 1. EROSION OF VILLUS MUCOSA AT VARIOUS INTERVALS POST MORTEM

Minutes after killing	Duodenum		Jejunum		Ileum	
	Fixed open	Fixed closed	Fixed open	Fixed closed	Fixed open	Fixed closed
10	(+)	++	−	(+)	−	−
20	++	++	−	(+)	−	−
30	++	++	(+)	+	−	−
50	++	++	(+)	+	−	−
70	++	++	+	+	−	−
100	++	++	++	++	−	−

Explanation of Symbols:
−, no erosion
(+), villus tips show limited (or slight) desquammation
+, villus cores exposed
++, epithelium lost on most of basal part of villus—heavy erosion

the duodenum and jejunum which had not been washed and which were not immersed in fixative for the first 30 minutes post mortem (Figs. 4, 5) exhibit loss of the epithelium of the tips. The erosion is much greater in the duodenum than in the jejunum; in the latter some villi appear unaffected.

When these various samples were sectioned, those which had been washed and fixed quickly showed separation of epithelium from the membrana propria to be considerably less (Fig. 6), while in the unwashed, delayed samples there was extensive separation of epithelium along the sides of the villi, and sloughing of cells at the tips was pronounced (Fig. 7).

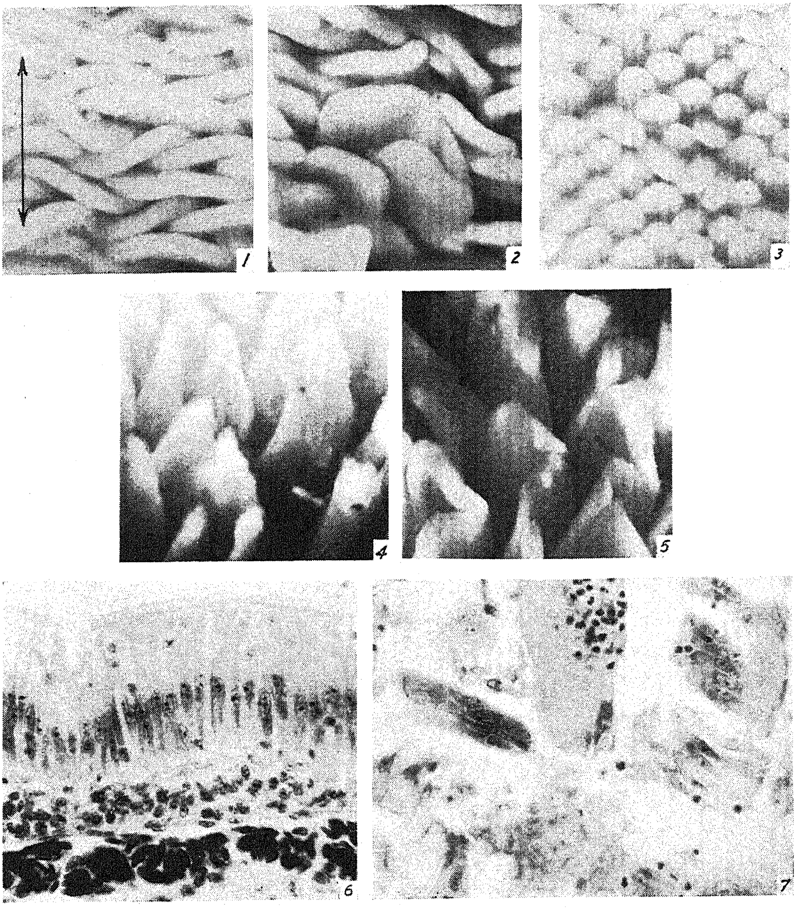

PLATE I

Figures 1, 2, and 3 were opened, washed and fixed flat within 10 minutes post mortem. Figures 4 and 5 were placed in fixative, unopened, 30 minutes post mortem and subsequently opened for photographing. The arrow indicates the antero-posterior axis of the intestine for Figures 1 to 5. Figures 1 to 5 are surface views taken thru water by reflected light. Note the difference in orientation of villus plates in the portions fixed flat, with those fixed closed. Sections were by the paraffin technic.

1. Duodenum, showing smooth contours of villus plates. ×14.
2. Jejunum, the same. ×14.
3. Ileum, as seen either after 10 minutes or 90 minutes post mortem. ×14.
4. Duodenum. The villus tips are highly abnormal with considerable loss of epithelial cells. ×14.
5. Jejunum. Only the very tips of the villus plates exhibit the desquammation of epithelium. ×14.
6. Duodenal villus tip, fixed immediately, sectioned at 10 μ and stained with Delafield's hematoxylin and eosin. ×384.
7. Duodenal villus tip after delayed fixation. ×384.

This technic of rapid versus delayed fixation was repeated on a second bird, all three portions of the small intestine being sampled. Three persons participated in the work so as to expedite the process and to make the results from the duodenum, jejunum, and ileum comparable. The findings from these latter preparations are summarized in Table 1.

Even the 10-minute sample of duodenum showed some slight loss of cells at the tips. This may have been due to the vigorous stream of warmed saline played upon the villi to remove debris, intestinal ferments and mucous.

From the data in Table 1 it is evident that the duodenal epithelium is most susceptible to post-mortem damage, and that a decreasing gradient of susceptibility to erosion occurs from the duodenum to the ileum. The ileum is able to retain its epithelium intact even when the application of fixative is long delayed.

The differential in susceptibility of epithelium in the several parts of the small intestine may be associated with two factors. In the first place, the concentration of digestive ferments is greatest in the upper part of the intestine. Secondly, the epithelium of the duodenum and upper jejunum is probably more loosely attached to the membrana propria than is that of the lower jejunum and the ileum; mitosis in the crypts of Lieberkühn indicates that epithelial replacement is greatest in the duodenum and diminishes thruout the remainder of the small intestine.

TIME SAVERS FOR FIXING AND DEHYDRATION

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ABSTRACT.—A double wash bottle for dispensing two-part fixing solutions is described. Equal volumes of each stock solution are delivered simultaneously into the same vial.

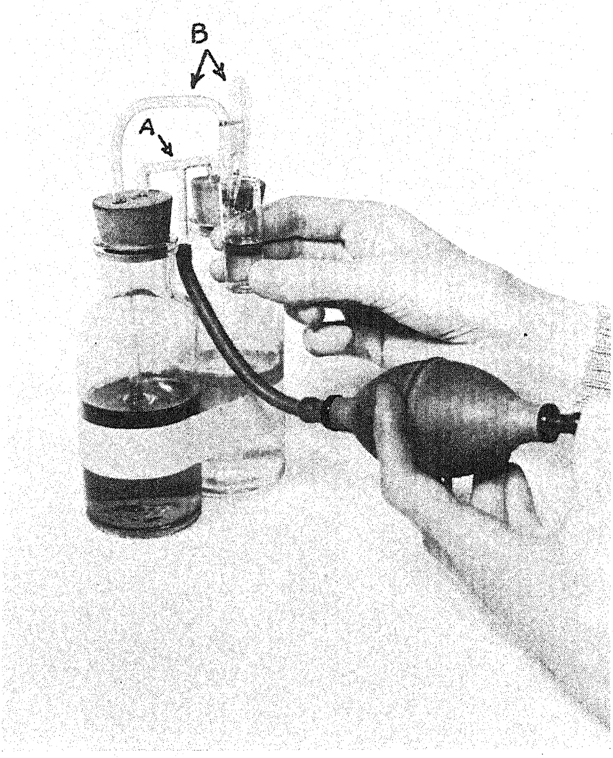

Fig. 1. Fixing-solution dispenser, showing use of thumb as valve when applying pressure.

A device for dehydrating tissues consists of a Buchner funnel closed at the bottom by a rubber tube and pinch clamp which facilitate changing the dehydrating alcohols. Tissues are placed in perforated brass baskets in the funnel.

[1]Maintained in coöperation with Yale University, New Haven, Connecticut.

The time-consuming labor of routine fixing and dehydration operations may be reduced by the use of several simple devices.

Most two-part fixing-solution formulae can be so modified that equal volumes of each stock solution are used, and are readily dispensed from the double wash bottle shown in Fig. 1 which saves time in stopper pulling and pouring. The accuracy with which equal

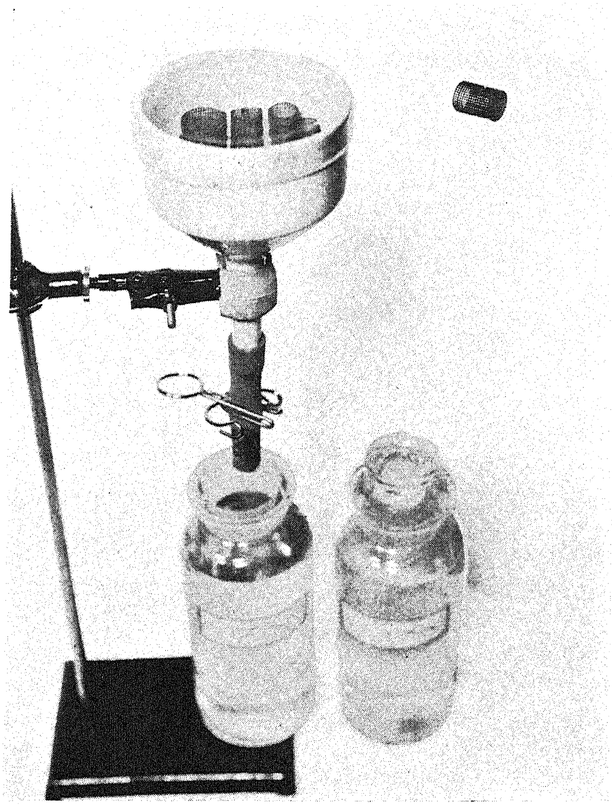

Fig. 2. Dehydration apparatus with brass basket at upper right. The Buchner funnel is covered with a Petri dish.

volumes of the stock solutions are delivered is much greater than that secured by the usual estimates, and is dependent on filling the stock bottles to the same level and making both delivery tubes of the same dimensions thruout.

The fixing-solution dispenser is made with two wide-mouthed 8-ounce bottles stoppered with two-hole rubber stoppers. The ends of a glass T (Fig. 1, A) are bent downward (or upward if it is more convenient to have the bulb above the delivery tubes) and inserted

in one hole in each stopper. The delivery tubes (Fig. 1, B) which pass thru the other holes are bent as shown and the tips drawn out. There are no valves in the bulb which furnishes the pressure, the thumb serving as a valve when the solutions are to be dispensed. The bottles and the delivery tubes are taped together.

If the routine calls for the filling of numerous shell vials held in a block, the dispenser can be modified by lengthening the delivery tubes and supplying the air from above (ends of T bent upward) either by bulb or by mouth as with the common type of wash bottle.

When fixation is complete, the tissues are transferred from shell vials to soldered cylindrical baskets (Fig. 2) made of brass stock with about one hundred 0.5-mm. perforations per sq. cm. and 1/64 in. thick, with the smooth side turned inward to avoid catching and damaging the tissues. A convenient basket for root tips and other small materials is 12 mm. in diameter and 16 mm. in height. Few of even the finest root tips are lost thru the perforations of these baskets, particularly if longer tips are taken than is customary with other procedures.

Tissues are washed in the baskets, which may be corked to retain buoyant material. The baskets are then transferred to a 9-cm. Buchner funnel covered with a tight-fitting Petri dish (Fig. 2). The stem of the funnel is held in a ring-stand clamp and fitted with a short rubber tube closed by a pinch clamp. The dehydration series is run thru the material by pouring the solutions into the funnel with the pinch clamp closed and then drawing the solutions off from the bottom at the end of the proper time interval. The baskets are generally not corked during dehydration. An interval timer is excellent for keeping on schedule, thus effecting a further saving of time.

Infiltration is also accomplished in the baskets which are immersed in xylol and blotted with paper toweling to remove all paraffin from the perforations after the tissues have been removed. The funnel system of changing reagents should be applicable to the method of running up root tips cemented to cards.

Acknowledgments for some of the foregoing ideas are due to Dr. W. P. Stockwell, of the California Forest and Range Experiment Station, and to Mr. Ernest Jund, of the Division of Genetics, University of California, Berkeley, California.

DELAFIELD'S HEMATOXYLIN AND SAFRANIN FOR STAINING PLANT MATERIALS

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Abstract.—An improved schedule is suggested for staining plant materials in Delafield's hematoxylin and safranin. Tissues are stained first in Delafield's hematoxylin. A short bath in acidulated water (1 or 2 drops concentrated HCl to 100 cc.) removes objectionable precipitates, and at the same time serves as a destaining agent. The acid bath must be followed quickly by a thoro wash in tap water, or dilute lithium carbonate solution, to restore the original dark blue color (made reddish in the acid bath) of the hematoxylin and to "set" the stain. Once the hematoxylin solution is satisfactory, none of the reagents ordinarily used will remove it—unless they contain acid. Tissues are counterstained in rapid safranin (5 drops analin in 100 cc. of 1% safranin 0 in 50% ethyl alcohol); this materially lessens the time necessary for staining. The safranin is destained in 50% ethyl alcohol (which does not affect the hematoxylin) until sharp differentiation is secured. If destaining is too slow, or differentiation poor, a quick rinse in acidulated 50% alcohol usually sharpens contrast of the stains. This must be followed quickly by a wash in 50% alcohol containing lithium carbonate to neutralize the acid. Dehydrate, and mount as usual. This schedule allows each stain to be individually, and independently, controlled at the will of the operator.

Delafield's hematoxylin is one of the most useful histological stains for the precise staining of cellulose cell walls. Methods of using this stain, however, have often yielded uncertain results, varying with the skill or luck of the technician. Delafield's hematoxylin should stain well, when used alone, except for characteristic precipitates that often appear in the tissues. An excellent double stain is Delafield's hematoxylin and safranin, but frequently this combination appears to cause trouble. Sharp differentiation is not always obtained, and objectionable precipitates often remain in the tissues after staining. The addition of safranin may unduly mask the hematoxylin in the tissues and cause a muddy stain. Staining may be a lengthy process with uncertain results in the finished slides. The difficulty in getting uniformly good results has caused many workers to dislike, or reject, this combination of stains. Nevertheless, when a well-stained slide is produced, these stains appear to justify the claims made for them.

Stain Technology, Vol. 15, No. 2, April, 1940

Schedules for Delafield's hematoxylin and safranin ordinarily recommend that tissues be stained in safranin first, at least several hours, and sometimes over night or longer. Some older schedules even suggest boiling freehand sections in safranin. After staining in safranin, the tissues are destained in 50% ethyl alcohol (sometimes acidulated) almost to the proper color; then Delafield's hematoxylin is applied. Within a few minutes the hematoxylin is usually dark enough, and a bath in acidulated water or alcohol is then used to differentiate the stains. The acid removes both stains from the tissues. Consequently, the interval allowed in each stain and the duration of the acid bath must be carefully timed or poorly differentiated tissues result. Obviously, the quality of the finished slide cannot be judged properly until after the final acid bath; if the stains are not then satisfactory, the whole process must be repeated—usually after decolorizing the tissues in acidulated water or alcohol.

When Delafield's hematoxylin is used in combination with eosin or erythrosin, however, the above procedure is commonly reversed; the tissues are first stained in the hematoxylin, then in the counterstain. Chamberlain[1] indirectly indicated that safranin could be used as a counterstain when he stated: "After the hematoxylin is just right, apply a contrast stain, if you wish to double stain." Later, however, he gave detailed directions (pp. 88–91) in which he recommended safranin first, followed by Delafield's hematoxylin. Recently, in staining meristematic tissues, Boke[2] used safranin dissolved in xylol as a counterstain following Delafield's hematoxylin.

The possibility that safranin could be used profitably as a counterstain *following* Delafield's hematoxylin, in the routine staining of plant materials, was suggested to the writer in 1931 by Professor R. B. Wylie; and experiments along this line were undertaken, using the following solutions:

Delafield's hematoxylin, made according to the formula of Stirling and Lee as given in Chamberlain (1932). Only fully aged, naturally ripened, stain was employed. Coleman and Bell hematoxylin was used, Cert. Nos. FH-5, and FH-11.

Rapid safranin, made by adding 5 drops of anilin to 100 cc. of a 1% solution of safranin O in 50% ethyl alcohol. The anilin acts as a mordant. National Aniline safranin O was used, Cert. Nos. NS-10 (dye content 94%) and NS-9 (dye content 97%).

Points favoring the use of these stains in this order may be briefly summarized as follows: (1) With the hematoxylin applied first, it is easier to get the stain just the shade of color desired. The bath in

[1]Chamberlain, C. J. 1932. Methods in Plant Histology, 5th Ed. University of Chicago Press. See p. 51.

[2]Boke, Norman H. 1939. Delafield's hematoxylin and safranin for staining meristematic tissues. Stain Techn., 14, 129–31.

acidulated water following the stain dissolves precipitates that so often accompany Delafield's hematoxylin, and also serves as a destaining agent. Once the hematoxylin is "set" in the tissues, none of the reagents ordinarily used will decolorize it, unless they contain acid. (2) When differentiating in 50% ethyl alcohol after counterstaining in rapid safranin, the counterstain is washed from cellulose cell walls faster that it is from lignified structures. Since this treatment affects only the safranin, it can be continued until the blue of the hematoxylin stands out in sharp contrast to the red of the safranin. Each stain may thus be individually controlled at the will of the operator. The use of rapid safranin greatly shortens the time of staining. Good results can be obtained with the usual safranin solutions, but the time of staining must be considerably increased.

The time of staining must be determined experimentally for various tissues. Ten minutes in Delafield's hematoxylin followed by a 15-minute stain in rapid safranin has been about right for sections of *Osmunda* rhizome killed in a mixture of formalin, alcohol and acetic acid (denoted, "F. A. A."). With most leaf sections, a 5–15 minute stain in Delafield's followed by 10–15 minutes in rapid safranin should give good results. Only tissues extremely difficult to stain should take more than 30 minutes in the hematoxylin. Tissues often stain poorly, or not at all, if the killing fluid has not been thoroly washed out before embedding. Delafield's hematoxylin stains especially well following killing fluids containing chromic acid, but a satisfactory stain can be obtained following practically any of the usual killing agents. If tissues are difficult to stain, a 5-minute bath in 1% aqueous potassium permanganate is suggested as a mordant.

The accompanying provisional schedule has been used with success for the past 7 years in classes in histological technic, and has proved effective for critical research materials, especially when careful measurements must be made of plant cell walls. Tissues stained in Delafield's hematoxylin and safranin are excellent for photographic purposes. The accompanying schedule, written for paraffin sections, may easily be modified for use with free-hand sections or bulk material.

Staining Schedule for Delafield's Hematoxylin and Safranin

1. Remove paraffin and get slides into tap water. All alcohol should be removed from tissues or staining may be spotty.

2. Stain in Delafield's hematoxylin—5 minutes to 1 hour. As staining is progressive, try minimum time first, then increase by multiples of 5 minutes—up to 30 minutes. If tissues are unaffected

after 30 minutes, continue staining for a total of one hour. If tissues are unstained after one hour, look for causes of failure other than the hematoxylin.

3. Rinse well in tap water to remove surplus stain.

4. Examine under the microscope. Sections at this point should be somewhat overstained. Destain in acidulated tap water (1 or 2 drops concentrated hydrochloric acid in each 100 cc. water). Gently agitate slides in acidulated water until sections assume a reddish color—usually in 3–10 seconds. Time in acidulated water must be varied according to density of stain desired, type of tissue, and the concentration of acid used. The acid destains the tissues, and dissolves objectionable precipitates. Because these precipitates so frequently occur, the acid bath should always be used. (If the color is too light, thoroly wash out all acid in tap water and restain in the hematoxylin.)

5. Wash *immediately* in clean tap water. Allow to stand in several changes of tap water until sections assume their original dark blue color. If the blue color does not soon reappear, or if a more positive method is desired, allow slides to stand in 0.1% aqueous lithium carbonate until the color is restored. Five minutes should be sufficient. The carbonate provides the necessary alkaline medium for the hematoxylin, and often intensifies the stain. Use a weaker carbonate solution if the sections persistently assume a hazy, light blue color. The lithium carbonate solution may macerate certain soft tissues. If this occurs: (1) add 5 or 10 drops of 0.1% lithium carbonate solution to a Coplin jar of tap water, (2) add 3–4 drops of 5% ammonia to the tap water, or, (3) use plain tap water (usually slightly alkaline). The color is usually darker when lithium carbonate is used than it is when plain tap water is employed.

6. Rinse in tap water to remove carbonate solution. Examine under the microscope and repeat steps 2, 3, 4, 5, and 6, if necessary, to get proper density of stain. If color is too light, either shorten the time in acidulated water, use less acid, or stain longer.

7. Pass slides thru alcohol series to 50% alcohol.

8. Stain in rapid safranin—5 minutes to over night. Try minimum time first, then increase in multiples of 5 minutes—up to 30 minutes. Usually, difficult tissues will stain in 1–4 hours; rarely should it be necessary to stain over night.

9. Drain, blot, or rinse off (with slightly used or clean 50% ethyl alcohol) the excess safranin, and differentiate in clean 50% alcohol. This destains the safranin only, and should be continued until sharp differentiation is obtained.

10. If destaining of safranin is too slow, or differentiation poor, dip and gently agitate the slide in acidulated 50% alcohol (1 or 2 drops hydrochloric acid in 100 cc. alcohol) for a few seconds. This quickly removes the excess safranin, and slightly reduces the hematoxylin, but usually sharpens contrast between the stains. Repeat if necessary.

11. Wash *immediately* in clean 50% alcohol made alkaline by adding 5–10 drops of 0.1% aqueous lithium carbonate to a Coplin jar of the alcohol. Allow slides to stand in fresh, alkaline, 50% alcohol for at least 10 minutes before dehydrating.

12. Dehydrate, clear, and mount in neutral balsam.

Note: To stain in Delafield's hematoxylin only, omit steps 8, 9, 10, and 11.

Whenever a metallic-appearing scum forms on the surface of the hematoxylin it should be filtered, or skimmed off with the torn edge of a piece of filter paper. Delafield's hematoxylin may be used full strength or, preferably, diluted with distilled water. The writer ordinarily dilutes the hematoxylin with one-third its volume of distilled water. Greater dilutions may be used, usually with more precise results, but the time of staining must be increased accordingly. Dilution of the safranin with 50% alcohol may be necessary for certain tissues, but, as with the hematoxylin, the staining is often more precise. A few experiments will determine the best procedure for each lot of new material.

A SIMPLE STAINING METHOD FOR HISTOLOGY AND CYTOLOGY

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In 1937 Cannon[1] called attention to the possible histological uses of a greenish-black dye put out by the British Dyestuffs Corporation under the name chlorazol black E. This proves to be an acid dye of the azo group, (Colour Index No. 581), a sulfonated triazo dye, which has been sold by various manufacturers under a variety of names. The National Aniline and Chemical Co., upon request to furnish a sample of this dye, have supplied the product which they sell under the name Erie black GXOO; I. E. Pont deNemours and Co. in response to a similar request furnished a sample labeled pontamine black E. These two samples have been submitted to spectrophotometric analysis and appear to be almost identical, altho Erie black GXOO is slightly bluer than pontamine black E.

Apparently this dye should be better known. The writer has tested 1% solutions of both samples in 70% alcohol, freshly prepared and unfiltered, on histological and cytological material, and has found them to give excellent pictures, both samples responding similarly. The method of staining is as follows:

Run paraffin sections down thru water. Stain 5–10 minutes (rather than 15–30 minutes as suggested by Cannon) in a 1% solution in 70% alcohol, freshly prepared and unfiltered.[2] (Cannon calls for a saturated solution of dye in 70% alcohol). Drain off excess dye, wash in 95% alcohol, absolute alcohol, xylene, and mount. No mordant and no differentiation are required.

In the epithelium tissue of a rabbit embryo (Zenker's fixation) the cells were definitely outlined black; the chromatin, dark; cytoplasm, greenish gray; and the nucleoli, black. The muscle fibers and lymphocytes were a decided black, and the blood cells, a yellowish green.

Zenker-fixed kidney sections and Bouin-fixed sections of intestine stained in varying shades of green, gray and black, with the blood cells of the kidney sections a light green.

Sections of *Puccinia* on aster (fixed in Allen's B-15 fluid) showed a jet black outline of the cell walls; the cytoplasm and plastids, grayish

[1]Cannon, H. J. A new biological stain for general purposes. Nature, **139**, **549**. **1937**.

[2]In the present work staining periods of 1, 5, 10, and 15 minutes were tried.

green; and the nucleoli, dark green. The infected part and the hyphae stained yellowish green.

A cross section of a fern leaf (Flemming fixation) stained the various structures as follows: cell walls a definite black; the epidermis walls, heavy black; nuclei green, with nucleoli a dark green; cytoplasm light amber; plastids gray. The suberized walls of midrib and veins were dark amber.

In sections of an onion root tip (Flemming fixation), the cell walls stained dark gray almost black; cytoplasm, grayish green; nucleus a yellowish green, with the nucleoli a deep yellow (amber).

This stain certainly shows sharp differentiation for either histological or cytological purposes, and merits wider use for general purposes.

CHLORAZOL BLACK E AS AN ACETO-CARMINE AUXILIARY STAIN

B. R. Nebel, *Geneva, N. Y.*[1]

Abstract.—In making chromosome counts on plants and plant parts treated with colchicine it was found that in cases where aceto-carmine alone is not satisfactory—as in axillary buds of apple, pear, plum, peach, apricot, and cherry—the following method was effective: Dissect out the meristematic parts of the axillary bud under a binocular (or cut free-hand sections) and transfer the dissected tissue immediately to a solution of 3 volumes alcohol to 1 volume acetic acid for killing and fixing. Let the fixative act at least 10 minutes; a longer time, 12-24 hours, improves the staining quality. Wash in at least 3 changes of 70% alcohol to remove most of the acid. Stain for 5-25 minutes in 1% chlorazol black E[2] in 70% alcohol. Rinse in 3 changes of 70% alcohol to remove excess stain. Transfer the material to a slide, cover with a drop of aceto-carmine, and if necessary, dissect further under a binocular. Cover with cover glass, heat, flatten and seal, or run Zirkle's fluid under the cover for permanent mounting. For smears of sporocytes, chlorazol black E may also be employed alone, or in combination with aceto-carmine, if a dark purple nuclear stain is desired.

The inducing of polyploidy in deciduous fruit trees and herbaceous plants by the use of colchicine and other chemicals depends to a large extent on valid methods of tracing the progressive action of the drug in the tissue and later in detecting tetraploid tissue areas on diploid plants. In deciduous fruits various concentrations of colchicine in 5% alcohol were applied to axillary buds. The penetration of the drug into the meristems of the bud and its action thereupon were followed by successive dissection of treated buds. The success of the treatment was gauged by dissecting axillary buds laid down 1-3 months after the first treatment, that is, after growth and shoot development had occurred from the bud first treated.

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[1]Approved by the Director of the New York Agricultural Experiment Station as Journal Paper 349. Dec. 7, 1939.

[2]Attention was called to this dye by the Biological Stain Commission; see preceding article by Darrow. The sample used in this work was obtained from The National Aniline and Chemical Co. under the name Erie black GXOO; the DuPont Co. uses the name pontamine black E. All three of these names are regarded as virtual synonyms by the Stain Commission. Since the work was completed, this dye has been put on the certification basis by the Commission.

In vegetative tissue, nuclear stages are difficult to observe and chromosome plates were not readily stained with aceto-carmine in deciduous fruits. The following method was found useful to overcome this difficulty.

Axillary buds were prepared for staining by cutting from the main stem or branch as in nursery budding. Such buds may be transported in moist dishes and kept for one hour without the loss of mitotic cells in the meristems. The use of crushed ice during transport and storage is advisable.

The following procedure may be modified to satisfy individual needs: An apple bud, for example, was placed in a drop of water on the stage of a binocular, dissected from its woody base, then peeled to free the central axis or axes carrying the meristematic leaf and shoot

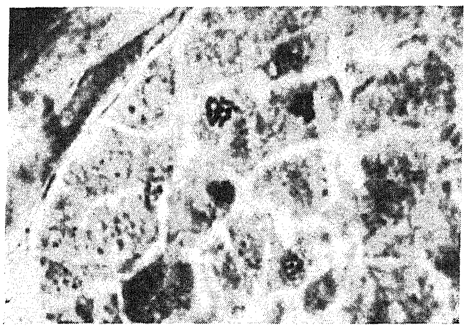
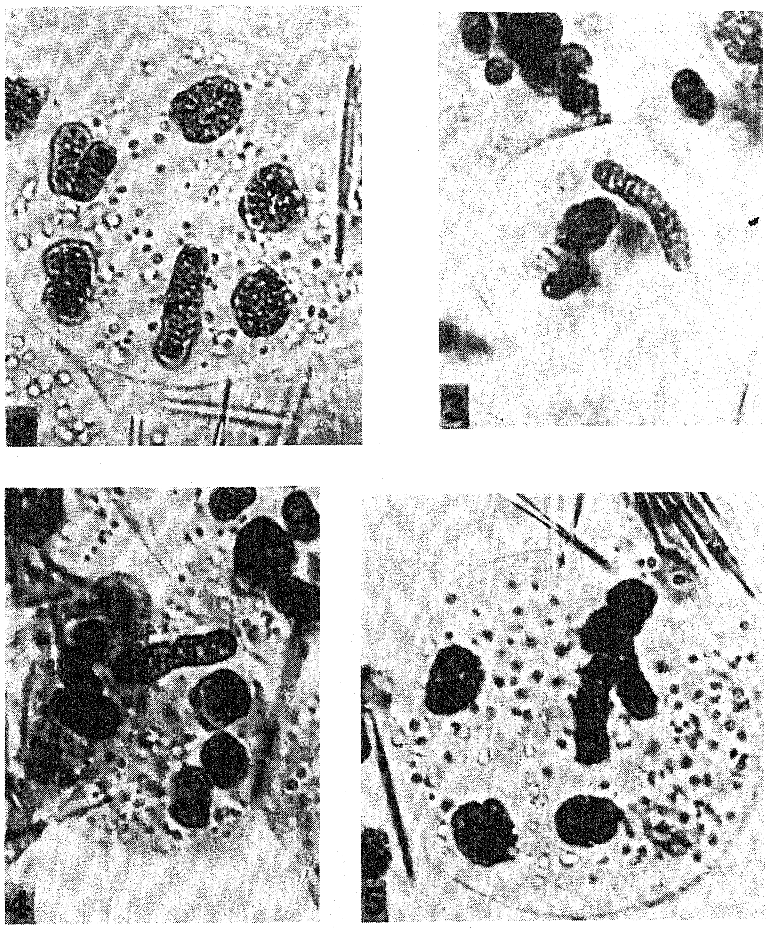


Fig. 1. Tissue of a pear bud treated with colchicine for 5 days with 3 daily applications and then allowed to recover for 2 days.

initials. When only the desired parts remained, the axis or axes were cut longitudinally and quickly immersed in 3:1 alcohol and acetic acid for killing and fixing. Better differentiation and more contrasty staining were achieved with material which remained in the fixative over night, than with fixation for 5–10 minutes only. When speed was essential, routine results were obtained with short fixation. After fixing, the material was transferred to three changes of 70% alcohol to remove the acid from the tissue. It was found inadvisable to cut the total time in 70% alcohol to less than 5 minutes.

The stain was prepared by dissolving 1% by weight in 70% alcohol and filtering thru a coarse filter. The stain is somewhat colloidal and tends to clog a fine filter. Small brushes were used for transferring the material. The exact time for staining had to be tested for each species of plant; in general, it should not be less than 5 minutes or more than 25 minutes.

The period of staining was followed by rinsing in three changes of 70% alcohol. The material was then transferred to a slide carrying a small drop of aceto-carmine, and all non-meristematic parts were



Figs. 2–5. Sporocytes of *Tradescantia reflexa* in first meiotic division. All exposures were identical, on Wratten M blue label plates developed at 65° for 12 minutes in D76; and printing was identical in all cases.

Fig. 2. Aceto-carmine after fixation in 3:1 alcohol-acetic-acid.
Fig. 3. Carmine, followed by chlorazol black E; mounted in Zirkle.
Fig. 4. Carmine, and chlorazol black E; mounted in aceto-carmine.
Fig. 5. Chlorazol black E only; mounted in 45% acetic acid.

carefully dissected away under a binocular. With the smallest amount of material finally remaining on the slide, results were found most satisfactory. The preparation was then covered, heated, lightly

squeezed and examined. If permanent mounts were desired, the McClintock technic was used or the aceto-carmine was gradually replaced by Zirkle's fluid.

The visual impression obtained from examining the slide before applying the aceto-carmine was that no appreciable amount of stain had been taken up by the chromatin. However, after the use of both stains, the chromosomes are stained sharply as they would not be with aceto-carmine alone. This is illustrated in figure 1 representing pear tissue.

While, in the present investigation, chlorazol black E was thus used primarily to stain tissues in which chromatin could previously not be stained quickly, a series of slides was made to test the new stain on *Tradescantia* sporocytes which have a high nuclear dye affinity.

The anther content was smeared on a clean slide and covered with a large drop of alcoholic acetic acid (1 volume glacial acetic to 3 volumes alcohol). After 1 minute, the slides were transferred to Coplin jars containing 70% alcohol. From there on the slides were treated differentially as illustrated in figures 2 to 5. Figure 2 is the check, the material being stained and mounted in aceto-carmine. This resulted in high transparency with a high density range within the chromosomes which is often confusing to the eye. When chlorazol black E was used after carmine and the material was then mounted in Zirkle's fluid, the density of the chromatin stain was intermediate and well adapted to visual work (Fig. 3). In this preparation the cells were neither swollen nor flattened. The material in Fig. 4 was treated with carmine before and after staining with chlorazol black E, and was mounted in carmine. The swelling was intermediate and the cells were flattened in this procedure. The chromatin was densely stained.

Finally chlorazol black E was used alone and the material mounted in clear 45% acetic acid without carmine. The chromatin stain was very dense and well differentiated (Fig. 5). The cells were much flattened.

Thru the courtesy of Mr. J. D. Nantz of the National Aniline and Chemical Co., two black purified derivatives of chlorazol black E were used on *Tradescantia* sporocytes. Both of these stained nuclear material after acid fixation only a weak gray so that they appear to have no value for the present purpose. The names of these dyes were: Buffalo black NBR and durol black 2 B.

In conclusion: The ease of applying chlorazol black E, its high nuclear affinity and the fact that it appears relatively stable in acid solution commend the stain for general use.

A TECHNIC FOR STAINING MOUSE PITUITARY

EARL B. SCOTT, *Department of Biology, University of Pittsburgh*

Many technics for histological staining of the pituitary gland have been devised. Since most of the methods described have been for animals other than the mouse, and since the mouse hypophysis, for some unknown reason, is difficult to stain by the accepted technics, the present writer has worked out a modification of Mallory's triple staining method.

Removal of the Pituitary. After the skull cap has been removed, the brain is loosened at the anterior end and carefully reflected back. The hypophysis is now exposed. The point of a wet needle is then used to loosen the capsule of the gland, which comes away easily, and a wet scalpel is slid under the pituitary. In this manner the gland can be easily lifted from its shallow sella. Sometimes the hypophysis folds itself double on the scalpel, but it can be very easily straightened out with a wet needle. It is important that the needles and scalpel be wet.

Fixation and Embedding. The pituitary is fixed in Zenker-formol for 24 hours and washed in running water for 24 hours. The tissue is then dehydrated in two changes of cellosolve[1] and infiltrated and embedded in Hance's rubber paraffin.[2]

Staining. The following steps are used in staining:

Xylol; cellosolve; cellosolve-iodine solution, 10 min.; cellosolve; distilled water, (two changes); Mallory's 0.5% acid fuchsin,[3] 5 min.; distilled water (two changes); 15 min. in Mallory's Solution 2 (aniline blue,[3] 0.5 gm.; orange G, 2.0 gm.; 1% phosphomolybdic acid, 100 cc.); distilled water (two changes); cellosolve (a dip or two); distilled water; Mallory's Solution 2, 1½ min.; distilled water (two changes); cellosolve (two rapid changes); oil of thyme and cloves; xylol.

By using the above method, acidophiles were stained a brilliant red, basophiles blue, and the chromophobes were unstained. The same method was used in staining cat hypophyses with excellent results. In the cat, negative images of the Golgi apparatus were evident.

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[1]Inkster, R. G. The use of 'Cellosolve' for rapid dehydration in paraffin embedding and in staining of sections. *J. Path. and Bact.*, **44**, 269. 1937.

[2]Hance, R. T. A new paraffin embedding mixture. *Science*, **72**, 253. 1933.

[3]Coleman and Bell dyes were used by the writer in this technic.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi *Abstract Editor*

MICROSCOPE AND OTHER APPARATUS

FEDER, J. M. **Adaptation of the rolls razor to a new type of microtome blade.** *J. Lab. & Clin. Med.*, 25, 202–4. 1939.

The blade of the rolls razor is made of high quality English steel. It is hollow ground and readily honed and stropped. To adapt it for use on a microtome, a holder made from a piece of tool steel approximately the size and shape of a microtome blade (12.5 cm. long and 0.5 cm. thick) is recommended. The holder is 2.9 cm. wide, and at the thinnest portion 0.2 cm. thick, being tapered to a blunt edge. In the center of this bar, a slot 3.8 cm. long and 1.5 cm. deep is cut to hold the blade. On the left end of this slot, two pins engage in the two depressions on one end of a rolls razor. The opposite end of the blade is fitted with an inset steel ball. A set screw with a knurled head is placed in the right end of the holder and so arranged that when tightened its inner edge engages the ball of the blade making it immovable. The blade is placed in the depression so that the two small pins engage in the proper depressions and the set screw is tightened. The blade proves to be safe, efficient and economical.—*John T. Myers.*

HERCIK, FERDINAND. **Die Fluoreszenzmikroskopische Analyse der α-Strahlenwirkung.** *Protoplasma*, 32, 527–35. 1939.

For studies of the effect of alpha-radiation the author takes advantage of the fact that epidermal cells of *Allium cepa* become fluorescent, without any sign of injury, when immersed in an aq. solution (1:1000 dist. water kept at pH 5.8 with HCl) of potassium fluorescein (uranin) as determined by Strugger (Flora, **132**, 253, 1938).

Within 2–5 min. the healthy cells give a beautiful yellow green fluorescence by ultraviolet light. This first appears in the cell walls, then disappears to appear in the protoplasm and cell nuclei. The source of the alpha-radiation is a polonium preparation, the strength of which is previously determined by means of a Geiger counter. When tissues previously exposed to the radiation are immersed in the solution of fluorescein, the fluorescence appears in varying degrees, the blue-green illumination appearing, in some cells, only in the protoplasm, and in others in the vacuolar sap with varying intensities. The author claims the degree of fluorescence to be a far more delicate indication of damage to cells than the staining reaction with erythrosin.—*Robert Chambers.*

NEUWEILER, N. G. **Darkground illumination and Rheinberg colour discs—Some new and simple ideas.** *The Microscope,* 3, 81–2. 1939.

The simplest method of securing satisfactory darkground illumination is to insert suitable stops in the stop-holder of an Abbé condenser. This scheme works well at low and medium magnifications, but with a high-power objective some glare appears. The difficulty may be met by inserting the stops between first and second lenses of the condenser. Rheinberg color discs, which produce color effects with many microscopic objects, may be similarly inserted into the condenser.—*C. E. Allen.*

PIEKARSKI, G., und RUSKA, H. **Über mikroskopische Untersuchungen an Bakterien unter besonderer Beruchsichtigung der sogenannten Nucleoide.** *Arch. Mikrob.*, **10**, 302–21. 1939.

By means of the electron microscope (described by: E. Ruska, *Zts. f. Phys.*, **87**, 580, 1934; B. v. Borries and E. Ruska, *Wiss. Veröff. Siemenswerke*, **17**, 99, 1938) exceptionally fine photographs were obtained of various bacteria at magnifications ranging from 5,000 to 13,000 diameters.—*Merritt N. Pope.*

MICROTECHNIC IN GENERAL

(ANONYMOUS). **Nevillite V. Perfect substitute for balsam.** *El Palo Alto News*, **4**, 10–1. 1939.

Canada balsam mounts become yellow upon aging and turn decidedly acid in less than a year. A new synthetic resin, nevillite V, appears to answer the requirement of technicians for a thoroly reliable mounting medium. Nevillite V is a naphthene polymer, a cycloparaffin, available in the form of colorless clear lumps. Its outstanding characteristics are: it is inert, homogeneous, resistant to the action of light, also of dilute acids and alkalies; its acid number is negligible; M. P. 155° C.; it is soluble in hydrocarbons, including paraffin; it is insoluble in water, and alcohols. Toluene appears to be the most satisfactory solvent. For general purposes one should use a solution of 60% by weight, which yields a thinner fluid than the average balsam-xylene medium. The advantages of using nevillite V are that there is less solvent to evaporate, coverslips are more easily applied, and there is far less chance of trapping air bubbles in the mount.—*J. A. de Tomasi.*

ARMITAGE, F. D. **Dioxan in microscopical technique.** *The Microscope*, **3**, 212–6. 1939.

Dioxan is recommended in the preparation of fixatives. Tissues fixed in reagents insoluble or slightly soluble in dioxan should be well washed in water. Those fixed in readily soluble reagents can be washed immediately in dioxan. The following schedule is recommended for mammalian testes: Fix in Bouin's solution, 12–24 hr.; wash in 3 successive lots of 100% dioxan, 1 hr. each; change to fresh dioxan, and leave until required; place in equal parts dioxan and paraffin (m.p. 52°), 2–4 hr.; warm gently; shake gently; place in second dioxan-paraffin bath, 1 hr.; pure paraffin, 2–4 hr.; fresh paraffin, 1 hr.; imbed.

For mounting, either Canada balsam or the following dioxan-camsal-sandarac medium is satisfactory: Dissolve 25 g. gum sandarac in excess of dioxan; filter thru glass wool; evaporate filtrate to thick syrup, preferably on an oil bath; rub together in a mortar 3 g. salol and 2 g. camphor, heating gently to liquefy if necessary; add 1 cc. of this "camsal" to each 20 cc. of sandarac syrup; evaporate, if necessary, to the usual xylol-balsam consistency.—*C. E. Allen.*

CARLETON, H. M., and LEACH, E. H. **An improved method for flattening out paraffin sections.** *J. Path. & Bact.*, **49**, 572–6. 1939.

A method is proposed which is claimed to be particularly valuable when fixation has been faulty or post mortem changes are present. Sections prepared by this technic are superior to controls flattened on water, and closely approximate conditions in living cells and tissues. The cast block must be water-free. Sections are treated as follows: Put a small drop of diacetin (glycerol diacetate) on a clean slide. Do not albuminize the slide. Spread the drop with a section-lifter over an area at least as big as the flattened section will be. Put the section on the diacetin, place slide on hot plate, and help section flatten with aid of needles. Wipe off excess diacetin. Stand vertically in drying oven at 37° C., or, better, at 50°. After a few minutes more diacetin can be wiped off. After 12 hr., or preferably 1–2 days, remove and allow to cool. Sections stored in an oven for a few weeks become diacetin-free and damaged. Add xylene. After removal of all paraffin, add 0.1% celloidin in equal parts abs. alcohol and ether. After wiping off excess, allow celloidin to set, but not to dry completely (10–20 sec.). Plunge into 90% alcohol and leave for few minutes. Run down to water, stain as desired, dehydrate in graded alcohols. As celloidin is soluble in abs. alcohol, use 96% alcohol for differentiation. Place in abs. alcohol the shortest time possible

for dehydration or sufficient removal of celloidin; lower gently into xylene and leave 2 min. undisturbed; mount.—*S. H. Hutner.*

COLE, W. C., and SMITH, F. R. **A microscopic technique for studying fat globules in dairy products and other oil in water emulsions.** *J. Dairy Science*, 22, 420–1. 1939.

The method used for examining fat globules in milk, cream, chocolate milk, ice cream mix, evaporated milk and mayonnaise is: Dilute the material to be examined in dist. water (usually 1 to 100). Place approximately 0.02 ml. on a clean slide. Add about 0.01 ml. of an alkaline aq. solution of 1% nile blue sulfate ($NaHCO_3$ to make alkaline), and mix well. Place cover slip over mixture, and examine under microscope.—*H. Macy.*

DERBY, J. T. **A substitute for ethyl alcohol.** *The Microscope*, 3, 243–6. 1939.

Cellosolve (ethylene glycol mono-ethyl ether) is suggested as a substitute for ethyl alcohol. The following fixing-imbedding schedule is recommended for plant tissues:

After fixing and washing in water for 12 hr., dehydrate in cellosolve in the following dilutions (aq. solutions), 3 changes per day: 15%, 35%, 50%, 65%, 75%, 85%, 95%, 100% (change the 100% once). For the finest cytological details, a closer series may be desirable. Clear in xylene in the following combinations: 25% xylene, 75% cellosolve; 50% xylene, 50% cellosolve; 75% xylene, 25% cellosolve; 100% xylene (change the 100% once). Infiltrate with paraffin. Imbed.

Cellosolve may also be used instead of abs. alcohol in preparing material for venetian turpentine mounts.—*C. E. Allen.*

EARL, W. R. **Iron hematoxylin stain containing high concentration of ferrous iron.** *Science*, 89, 323–4. 1939.

A useful modification of Janssens' iron hematoxylin stain is suggested. Instead of the ferric salt, a mixture of equal parts of ferric and ferrous ammonium sulfate is recommended as follows: $Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$, 20 g.; $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 20 g.; hematoxylin dissolved in 25 cc. abs. methyl alcohol, 1 g.; glycerin, 25 cc.; water, 100 cc. Sections and whole mounts of tissue cultures show a transparent blue nuclear stain and require no differentiation other than soaking for a few minutes in a number of changes of dist. water, or in VanGieson picro-fuchsin for counterstaining.—*J. A. de Tomasi.*

KIRKPATRICK, J. and LENDRUM, A. C. **A mounting medium for microscopical preparations giving good preservation of colour.** *J. Path. & Bact.*, 49, 592–4. 1939.

An experimental study revealed that the fading of delicate dyes was correlated with the rapid acidification of the Canada balsam used. A study of a large number of natural and synthetic resins revealed that "distrene 80", a polystyrene of molecular weight approximately 80,000, obtained from the Messrs. Honeywill and Stein of London, was superior to any of the others. It dissolves in xylene; but as this solution in drying retracts badly under the coverslip, it is recommended that 7.5 cc. of tricresylphosphate be added to 40 cc. of xylene. In this quantity of the solvent 10 g. "distrene 80" should be dissolved. No acidification and no fading have been noticed with this mounting medium; it is cheaper than Canada balsam, and its melting point is much higher.—*S. H. Hutner.*

LAPIN, W. K. **[On the possibility of replacing Oleum caryophylorum in cytological practice with some other essential oils].** *Works All-Union Scient. Res. Inst. Humid Subtropics*, 1, Series 4, 75–8. 1937.

In searching for a substitute for clove oil as a differentiating agent in the Newton's gentian violet method, twelve different essential oils produced in Russia were tested on sections from root tips of *Poncirus trifoliata, Ficus carica,* and *Zea mais.* Imported clove oil (no source indicated) served as a control. With materials requiring a normal differentiation, best results were obtained by using the oils extracted from *Andropogon citratus, Manardia* and *Citrus salicifolia.* Immediate differentiation was obtained with oils from *Lavandula spica, Lavandula lanata* and *Citrus bigaradia.*—*J. A. de Tomasi.*

LILLIE, R. D., and EARLE, W. R. **Iron hematoxylins containing ferric and ferrous iron.** *Amer. J. Path.*, **15**, 765–70. 1939.

Studies of Janssens' iron hematoxylin (Stain Techn., **14**, 53–4, 1939) were extended to include the effect of ferrous iron on its staining and keeping qualities. Solutions which were made up with a high concentration of $FeSO_4$ remained usable for about 1 year while those without any, or with low concentration, spoiled in a few hours. The following staining solutions are recommended: (A) $FeNH_4$ $(SO_4)_2$ (violet crystals), 15 g.; $FeSO_4$, 15 g.; dist. water, 100 cc. (B) Hematoxylin, 1 g.; alcohol (95%), 50 cc.; glycerol, C.P., 50 cc. Mix A and B in equal quantities. The staining mixture is a nuclear stain and did not prove satisfactory for staining myelin sheaths in brain.—*H. A. Davenport.*

MONNE, LUDWIK. **Polarisationsoptische Untersuchungen über den Golgi-Apparat und die Mitochondrien männlicher Geschlechtszellen einiger Pulmonaten-Arten.** *Protoplasma*, **32**, 184–92. 1939.

By using as intense an illumination as possible (panphoto microscope of Leitz with a strong low voltage lamp) the author was able to observe with crossed nicols that the Golgi-apparatus especially of *Helix pomatia* is doubly refractive. This condition was intensified by replacing the isotonic (0.7–0.8%) salt solution as medium for a more concentrated one (1–2%). Better results were obtained by staining with rhodamin B and 6G, but the cells stained with chrysoidin were best. With a sufficient concentration of chrysoidin (concentration not given) the Golgi-apparatus shows up beautifully white against a black background. The same occurs with the stained mitochondria and lipoid layers and fibrillae which, however, do not light up if unstained. In spermatocytes and spermatids the golgi elements are straight or curved rodlets surrounding the centrosome. Between crossed nicols the rodlets successively light up by rotating the nicols.—*Robert Chambers.*

NIKLITSCHEK, A. **Das Tuschpräparat.** *Mikrokosmos*, **33**, 27. 1939.

The India ink method, discovered by Ehrenberg (1838), is fully reviewed. The ink may be rubbed up with dist. water in a depression of a white porcelain plate using a clear glass rod. Addition of ink should stop when the color of the ink creeping up on the margin changes from brown to jet black; trituration, however, should continue for at least 15 min. longer. The following applications are discussed:

Dry method: For blood parasites, a drop of blood is mixed with a drop of the ink on one side of the slide. The smear is then spread as in ordinary blood work, and allowed to dry. The preparation may be examined under oil immersion lens.

Wet method: For protozoa an equal amount of culture fluid and India ink are mixed. The criterion of a successful preparation rests in the proper distribution of the ink present. Immediately after placing the coverglass on the preparation, ring the mount with paraffin or Venetian turpentine. Cultures may thus be kept alive for a long time and studied at intervals.

When preservatives are added to the ink, they have a toxic effect upon the protozoa. This property may be advantageous in certain cases. When it is desired merely to slow the activity of protozoa, the addition of 0.5–4.0 g. of gum arabic in 10 ml. dist. water (that used in preparing the ink) will produce the desired results. Capsules of microörganisms may be clearly demonstrated. —*J. M. Thuringer.*

PASTERNACK, JOSEPH G. **A reliable one-hour method for the preparation of paraffin sections of tissues.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 8–13. 1940.

The following fixation and embedding methods are recommended: Fix tissue in boiling 10% formalin, 1 min. Rinse a few seconds in tap water. Cut 1–2 mm. Blot dry. Preheat the reagents. A desirable vapor pressure is obtained by screwing the lids on the containers as soon as the tissue is inserted. Place in abs. alcohol at 56° C., 10 min.; acetone U.S.P. at 56° C., 10 min.; xylene 2 changes at 56° C., 5 min. each. Put in paraffin at 60–62° C. in a vacuum container and hold at this temp. for 10 min. Embed in paraffin and put in ice water while the paraffin is still melted. Cut 5–8 μ. Float on water at 45–50° C. Affix to slide with Mayer's fixative. Wipe back and edges of the slide. Put on hot plate at

50° C. for 3 min. Gently pass over a micro-burner to melt the paraffin. Remove paraffin with xylene from a dropping bottle. Before xylene has entirely evaporated cover the section with 0.2% celloidin in abs. alcohol and ether (1:1). Drain off excess fluid. Blow briskly to hasten evaporation. Immerse in water a few seconds to harden celloidin.

The staining methods recommended are: (1) Weigert's iron hematoxylin, 3 min.; rinse quickly in water; Van Gieson's, 1 min.; 95% alcohol, 10 sec.; acetone U.S.P., 10 sec.; xylene, 10 sec.; mount in balsam. (2) Harris' hematoxylin, 1 min.; 1% ammonia water until blue; rinse quickly in tap water; eosin Y, 15 sec.; rinse in 95% alcohol; acetone U.S.P., 10 sec.; xylene, 10 sec.; mount in balsam.—*George H. Chapman.*

SAIER, ELEANOR, and COBURN, WILLIAM. **A modification of the dioxane dehydration method.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **18,** 71. 1938.

The authors have developed a modification of the dioxan dehydration method, using the following procedure: Fix in Zenker's fluid or 10% formalin; wash and trim; leave in 95% alcohol from the time received (noon or before) until afternoon of the same day; place in dioxan over night; paraffin, 2–3 hr.; 2nd paraffin, 2–3 hr.; embed, section, and stain. Blocks received by noon are ready to be sectioned by afternoon of the following day.

The dioxan may be recovered by decanting from the $CaCl_2$ and filtering with suction into a flask containing 10 g. anhyd. $CuSO_4$ per liter of filtrate. After standing several days, the filtrate is distilled fractionally, and the dioxan is recovered between 101° and 103° C. More than 50% of the dioxan may be recovered in this way.

In embedding and sectioning of surgical specimens, the above method is recommended for the following reasons: It has cut the routine embedding time from six to three days; it is slightly less expensive if the dioxan is recovered; it is easier to handle; in embedding blood clots and organs of small animals, it prevents the gritty hardness that results from the use of xylene.—*Jean E. Conn.*

TURNER, OSCAR A. **A manual of neurohistologic technique.** (Third installment in series). *J. Lab. & Clin. Med.*, **24,** 991–1003. 1939.

Procedures for the following staining methods are given:

(1) For connective tissue: Mallory's acid-fuschin–anilin-blue–orange-G stain; Haythorn's modification of Mallory's stain; Perdrau silver impregnation; Wilder method for reticulum; Karfield-Achúcarro tannin-silver method.

(2) For pituitary and pineal staining: Spark's method; Bailey's ethyl-violet–orange-G stain; Mallory's acid-fuschin–anilin-blue method; safranin–acid-violet stain; Hortega's stain for pineal parenchyma.

(3) For spirochetes: Dieterle method; Nieto's method; Jahnel's gold-silver method.

In addition to staining procedures, a discussion of the advantages and disadvantages of each method is included.—*Jean E. Conn.*

TURNER, OSCAR A. **A manual of neurohistologic technique.** (Fourth, and last, installment of series). *J. Lab. & Clin. Med.*, **24,** 1096–1108. 1939.

Details are given for a miscellaneous group of staining methods including calcium and iron in tissues, supravital staining for rapid tumor diagnosis, staining of Negri bodies, gross staining of the brain for anatomy or pathology, and frozen sections of complete organs. Standard formulae are given for a number of common fixing and staining solutions. The use of nitrocellulose as an embedding agent is discussed and tables of dye solubilities and specific gravity of alcohol-water mixtures are included.—*John T. Myers.*

DYES AND THEIR BIOLOGICAL USES

BANK, O., und BUNGENBERG DE JONG, H. G. **Untersuchungen über Metachromasie.** *Protoplasma*, **32,** 489–516. 1939.

For a study of coacervates, the authors investigated the nature of metachromatism, using toluidine blue and indigo carmine Ia as examples of basic and acid dyes respectively. Neutral violet, neutral red, janus green, nile blue,

brilliant cresyl blue, methylene blue, methylene green, and trypaflavin were also used as basic dyes (dye cation). As acid dyes (dye anion) orange G and erythrosin were employed.

The above-mentioned basic dyes were found to lose their metachromasy in dil. solutions but to become metachromatic in dil. solution when gum arabic or other negatively charged colloids are added. Inorganic anions may also induce metachromatism, especially ammonium paramolybdate and sodium phosphotungstate which give large complexes of polyvalent anions.

The disappearance of metachromatism is brought about by adding electrolytes with effective cations, the polyvalent cations being more efficient than the monovalent. An exception is the H-ion.

For acid dyes the metachromasy is enhanced by substances containing organic cations, e.g. clupein, chinin, strychnin, etc.

The authors conclude that metachromasy is a function of concentration of the dye in which aggregation of the dye molecules is facilitated when the concentration is high. The aggregation is due to van der Waal forces since the metachromasy disappears by the addition of alcohol, the effect increasing with the length of the carbon chain of the alcohol.—*Robert Chambers.*

BROH-KAHN, R. H. **The bacteriostatic action of sulfanilamide under anaerobic conditions.** *Science,* 90, 543–4. 1939.

The question of whether sulfanilamide acts as a bacteriostatic agent only in the presence of oxygen has long been a controversial point. The work reported in this paper substantiates claims that sulfanilamide may be bacteriostatic under anaerobic conditions. The evidence obtained from experiments with *Escherichia coli* may be summarized as follows: *E. coli* grows well aerobically in broth but poorly under anaerobic conditions; it is presumed to obtain energy partly from an aerobic, and partly from an anaerobic mechanism. If sulfanilamide inhibits only the aerobic mechanism, the degree of growth in the presence of oxygen and the inhibitor ought to approximate that obtained anaerobically—and such is the case. Sulfanilamide does not affect the action of glucose on growth obtained in the presence or absence of oxygen. It inhibits anaerobic growth in lactate-nitrate synthetic medium but does not affect aerobic cultures, thus displaying an activity similar to that of the cyanides. The conclusion is that bacteriostasis from sulfanilamide cannot be attributed to any non-specific mechanism, and that interpretation of its activity should be based upon performance under a variety of conditions in different media.—*J. A. de Tomasi.*

GERSHBERG, H., and FORBES, J. C. **Precipitation of insulin with rhodamine-B.** *Proc. Soc. Exp. Biol. & Med.,* 42, 95–6. 1939.

The authors have previously discovered that rhodamine B would precipitate pepsin, and later discoveries by others have shown that safranin precipitates insulin. Insulin is now found to be readily precipitated by rhodamine B at pH 7.2. The complex is dissolved in acid and the dye can be recovered with isoamyl alcohol, leaving insulin in the aq. layer. The insulin content of the washed ppt. formed on the addition of dye to a buffered solution of insulin has been demonstrated physiologically.—*M. S. Marshall.*

HOBBS, BETTY CONSTANCE. **The part played by bacteria in the reduction of methylene blue in milk.** *J. Dairy Research,* 10, 35–58. 1939.

The organism reducing methylene blue most rapidly in milk belonged to the coliform group, followed in decreasing order by *Streptococcus lactis,* and some fecal streptococci, *Staphylococcus aureus, Staph. albus, Staph. citreus,* some micrococci, group C hemolytic streptococci and some strains of *Streptococcus agalactiae,* and aerobic spore-formers. The methylene blue reduction in milk containing actively growing bacteria is considered by the author almost entirely the result of the metabolic reactions proceeding at the cell surface of the bacteria themselves. The methylene blue reduction test as used under practical conditions is considered a good index of the extent of bacterial metabolism in the milk.—*H. Macy.*

MODELL, W. **Chlorazol fast pink BKS as an anti-coagulant.** *Science,* 89, 349–50. 1939.

Clotting is a disturbing factor in kymograph experiments, where blood pressure of animals is to be recorded. Among many azo dyes, Chlorazol fast pink BKS

(C. I. 353) proves to be a particularly efficient anticoagulant, inexpensive as well as effective. Inasmuch as the crude dye (trade names: fastusol pink BBA; calcomine fast pink 2BL) is toxic it must be freed of salts and impurities. Purification is based upon precipitation from aq. solution by alcohol by the following technic: dissolve dye 1:15 in water, filter, add equal vol. of 95% alcohol; filter; wash ppt. with 70% alcohol; dry over steam and grind to a coarse powder. The yield is about 20% of the crude dye. A 5% solution should be used. A single intravenous dose of 100 mg. per Kg. prevents clotting for many hours. A method to avoid intravenous injection is proposed which calls for 0.5 cc. portions of the dye solution introduced at 30 min. intervals into the pressure system of the recording manometer just above the junction of the cannula and the rubber tubing.—*J. A. de Tomasi.*

NICHOLS, AGNES A. **Bacteriological studies of spray-dried milk powder.** *J. Dairy Research*, **10**, 202–30. 1939.

The methylene blue reduction tests at 37° C. and 55° C. were made on 405 samples of spray-dried milk. The weighted mean reduction time at 37° C. was 8.1 hr., varying from 3–14 hr. or more. At 55° C. the shortest time was 4.5 hr., and over 30% of samples required 12 hr. or more for decolorization.—*H. Macy.*

SMITH, W. S. **The excretion of phenol red in the dogfish, Squalus acanthias.** *J. Cellular and Comp. Physiol.*, **14**, 357. 1939.

The kidney tubules of the dogfish freely excrete phenol red; the maximum excretion being reached at a phenol red concentration of about 2 mg. per 100 ml. of plasma. Hippuran depresses the rate of phenol red excretion by the tubules, while creatinine has no marked effect.—*L. Farber.*

ANIMAL MICROTECHNIC

ARMITAGE, F. L. **A modified peroxidase stain for blood and bone marrow films.** *J. Path. & Bact.*, **49**, 579–80. 1939.

A method useful in distinguishing various cells in films of leukemic blood and sternal puncture blood marrow is as follows: Fix in 96% alcohol containing 10% formol, prepared immediately before use. Flood films with benzidine-H_2O_2 mixture (a filtered solution of 0.75 g. benzidine in 500 cc. 40% ethyl alcohol, with 7 cc. 3% H_2O_2 added and mixed immediately by shaking). Allow freshly-made films to stain 2 min., older ones longer. Control staining by washing with 40% alcohol until granular cells show definite yellow granules. Wash with 40% alcohol, abs. alcohol, dry in incubator. Counterstain with Leishman or dilute Giemsa. Wash with dist. water, blot dry.—*S. H. Hutner.*

HADJIOLOFF, A. **Coloration des lipides au moyen de solutions hydrotropes de Sudan et d'autres lipocolorants.** *Bull. d'Histol. Appl.*, **15**, 37–42. 1938.

Seeking to avoid the formation of precipitates of fat stains, and to avoid any loss of fat due to the action of the fat stain solvent, the author has turned to the use of aq. solutions of the stains of the Sudan series (Sudan II, Sudan III, Sudan IV, Sudan red, Sudan black, etc.). Stains were obtained from Holborn, I. G. F. and Ciba.

These aq. solutions are termed "hydrotropic solutions" after Neuberg, who showed that certain substances previously dissolved in dist. water have the property of bringing other insoluble substances into solution. Hydrotropic agents for the fat stains include soaps, caffeine citrate, caffeine benzoate, saponin, sodium trioleate, trichloracetic acid and sulfosalicylic acid. To make such a hydrotropic solution the fat stain is added to a conc. solution of the hydrotropic substance and kept in an oven at 56° C. for several days. After filtering once or twice, a perfectly clear solution results.

Frozen sections of formalin fixed material were employed. They were stained between 20 min. and 24 hr., carefully rinsed and mounted in glycerin.

An important fact from the histochemical point of view is that most of the fat stains change their color from red to deep blue in hydrotropic solution, in proportion to the increase in concentration of the hydrotropic agent used. For example, using sulfosalicylic acid as the agent, the color varies, with increasing concentration of the solution, from red, thru violet, to deep blue. Such a blue hydrotropic solution, however, upon entering fat or a fat solvent again becomes red. Thus

the author considers any red granules in a cell following treatment with a blue solution of one of these Sudan dyes as being truly of a fatty nature.—*M. Noble Bates.*

HEILBORN, O. **A new method of making permanent smears with special reference to salivary gland chromosomes of Drosophila.** *Lantbrukshögskolans Ann.* (Sweden) **4**, 89–97. 1937.

There are several drawbacks to the usual aceto-carmine smear technic for chromosomes: the tissue elements adhere poorly to the slide and are often lost when making permanent mounts; changes in the technic are practically impossible; the staining solution promptly weakens under the coverslip; the process of sealing and breaking the mount to make it permanent is not satisfactory. The following smear technic for *Drosophila* material dissociates fixation from staining: Dissect the larvae in a drop of Ringer's solution and prepare the salivary gland with two needles, of which one at least should be bent at a right angle. Transfer the glands to a drop of 50% acetic acid, fix 4–8 min., and remove to a dry slide. Cover with a coverslip wet with glycerin; hold it fast, and smear with the aid of a needle or a small roller. Suspend the inverted slide in 96% alcohol in a Petri dish until the coverglass drops off (in about 3 min.). Rinse in water. Stain overnight (15–25 hr.) in aceto-carmine. Wash in water, dehydrate, clear, and mount as usual. The method is fairly rapid, yields smears that are permanent from the beginning, and can also be applied to pollen mother cells.—*J. A. de Tomasi.*

LENDRUM, A. C. **A new trichromic staining method.** *J. Path. & Bact.*, **49,** 590–2. 1939.

The author modifies Masson's stain by replacing saffron, a dye of uncertain composition, with a solution of tartrazine in ethylene glycol monomethyl ether (cellosolve). The technic is as follows: Stain in haemalum; then in one of the following solutions: (A) eosin Y 0.1 g., erythrosin 0.1 g., phloxin 0.1 g., dissolved in 25 cc. of ethyl alcohol, and mixed with 25 cc. of 1% gallic acid containing 1% sodium salicylate; or (B) eosin Y 0.25 g., phloxin 0.25 g., dissolved in 35 cc. of 95% ethyl alcohol, with 15 cc. commercial formalin added. Stain in "A" 1–2 hr.; or in "B" 3-4 hr. Rinse with alcohol and add tartrazine cellosolve solution (a sat. solution tartrazine N.S., Imp. Chem. Ind., in commercial cellosolve). This replaces eosin with tartrazine. When the balance between red and blue is satisfactory, transfer to abs. alcohol, clear, and mount.

The best fixative is formol-sublimate (saturated aq. $HgCl_2$, 9 parts; commercial formalin 1 part, or 10% formalin followed by mordanting a few days in saturated $HgCl_2$). If the tissue is otherwise fixed or fails to show proper differentiation, leave unstained sections over night in formol-sublimate saturated with picric acid. After brief rinsing with water, iodine and $Na_2S_2O_3$, stain as above. For some tissues the tartrazine may be replaced advantageously by a solution of fast green F.C.F. in cellosolve.—*S. H. Hutner.*

LILLIE, R. D. **Some experiments with the Masson trichrome modification of Mallory's connective tissue stain.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **18,** 75. 1938.

The author reports on experiments undertaken in an attempt to shorten Masson's modification of Mallory's connective tissue stain without impairing the results. He reaches the following conclusions: (1) Formalin material may be satisfactorily stained by Masson's modification if sections are first mordanted with Bouin's fluid, picric acid or $HgCl_2$. (2) Mordanting for 10 min. at 58° C. in picric acid or $HgCl_2$ is as satisfactory as over-night mordanting in Bouin's fluid. (3) Various blue and green dyes of the sulphonated triphenylmethane group may be used satisfactorily as collagen stains.—*Jean E. Conn.*

FLINN, MACKAY. **A rapid staining method for opsonocytophagocytic indices.** *J. Lab. & Clin. Med.*, **25,** 316. 1939.

The stain is made by adding 0.15 g. methyl green and 0.5 g. pyronin to 15 cc. 95% alcohol, preferably in a mortar. The stain is completely dissolved by slowly adding 85 cc. of 3% phenol. This mixture should stand a week. Air-dried smears are flooded with the stain for 4 min., washed in tap water and air-

dried. The cytoplasm of the leucocytes is faint pink, the nucleus light red and the organisms deep red. As the cytoplasmic granules do not stain, confusion is avoided.—*John T. Myers.*

PRYCE, D. M. **Staining reticulocytes for demonstration purposes.** *J. Path. & Bact.*, 49, 594–7. 1939.

Permanent and relatively undistorted preparations of reticulocytes may be made as follows: Prepare a small quantity of sat. solution of cresyl blue in rigidly anhyd. abs. alcohol, and use while fresh. Dip grease-free slides in this solution and air-dry, or spread with another slide. Spread blood on this cresyl-blue slide, and cover blood film immediately with another slide (the "spreader" may be used) which has a strip of gummed paper attached to the under side at each end, thus preventing contact between the two slides but delaying evaporation. After 5 min. at room temp., remove covering slide, dry film, and apply Leishman's stain undiluted for 5 sec. to 5 min. Dilute and stain another 10 min. Wash off stain with dist. water until the slide just changes from blue to red. The water should be slightly acid. Leishman staining removes excess cresyl blue.

An alternative method is to treat the slide with cresyl blue for only 1 cm. at one end, place the drop of blood at this end and spread slowly so as to allow the blood to be colored blue. Hold the spreader in this position for 2 min., then complete spreading of the film, and dry. Reticulocytes stain in all parts of the film and excess stain is avoided.—*S. H. Hutner.*

PRYTHERCH, HERBERT F. **The life cycle and morphology of Nematopsis ostrearum, sp. nov., a gregarine parasite of the mud crab and oyster.** *J. Morphol. & Physiol.*, **66**, 39–66. 1940.

The author describes a rapid method of preparing smears and whole mounts of host tissues containing gregarine parasites. The procedure is as follows: Kill small seed oysters, previously kept out of water for several hours, by freezing them in the shell. Then remove pieces of expanded meats or mantle, fix in B3 (a modification of Bouin's solution containing urea and chromic acid), wash and dehydrate. Stain by flooding with pinacyanol dissolved in abs. alcohol (conc. not given), destain in lower alcohols, and stop at desired depth by immersion in water. Place slides in staining dishes containing sodium silicate for ½ hr. or more. Then apply cover glass and seal edges with marine glue.

The usual methods involving fixation in B3, and staining in Heidenhain's iron-hematoxylin, Mayer's haemalum, or Delafield's hematoxylin are also described.—*Elbert C. Cole.*

SHEEHAN, H. L. **The staining of leucocyte granules by Sudan black B.** *J. Path. & Bact.*, 49, 580–1. 1939.

To bring out the granules of leucocytes, the following technic is recommended: Dry blood film at least 15 min. before fixation, and fix ½ min. in methyl alcohol. Stain 1 hr. in sat. Sudan black B in 70% alcohol. Rinse in water, wash 1 min. with 70% alcohol to remove deposit of dye. Counterstain ½ min. with sat. solution "alcoholic" eosin in 70% alcohol. Rinse in water, stain 3 min. in sat. aq. methylene blue, rinse, blot dry. The granules in the leucocytes are deep black and are not decolorized by 70% alcohol or mounting in Canada balsam.—*S. H. Hutner.*

WALKER, THOMAS F., and SWEENEY, PATRICIA A. **A method of counting blood platelets.** *J. Lab. & Clin. Med.*, **25**, 103–4. 1939.

The following technic is proposed: Draw a 1.1% solution of sodium oxalate to the mark 1 in a white blood cell pipette and expel it. Draw the blood to mark 0.5, and the diluting fluid (1.1% sodium oxalate) to mark 11, and mix. Place a heavy rubber band around the pipette to cover both ends and centrifuge just enough to make the red cells settle (usually 30 sec. at 1600 R.P.M.), or let stand upright for about 2 hr. Gently expel the red cells by blowing. Place a drop of the clear fluid in the counting chamber. After 10 min., count all the platelets in 80 small squares, and add 3 zeros to get the number of platelets per cu. mm.—*John T. Myers.*

PLANT MICROTECHNIC

KASSANIS, BASILIOS. **Intranuclear inclusions in virus infected plants.** *Ann. Appl. Biol.*, **26**, 705–9. 1939.

Two kinds of intracellular inclusions in solanaceous plants infected with severe etch virus are described. One occurs in the cytoplasm and is similar to the X-bodies found in many other plant virus diseases. The other occurs only in the nuclei. These intranuclear inclusions appear to be crystalline, and have the form of thin rectangular plates.

The most suitable fixative for the intranuclear inclusions was found to be formol-saline (20 parts formalin, 80 parts 0.9% NaCl solution). Fixatives containing acetic acid, picric acid or alcohol do not give good results as they coagulate the proteins. Also, the first dissolves the crystals. Flemming's solution without acetic acid gives good results. The crystals stain readily with acid dyes such as acid fuchsin and eosin. The simplest method for differentiating the intranuclear crystals is to mount an epidermal strip in an aq. solution of eosin. The best method for staining fixed preparations is that of Kull, using acid fuchsin, toluidine blue and aurantia, which was used by Paillot (1926) in working with the polyhedral disease of silkworms. The crystals and the nucleoli stain red, and the chromatin blue. With Heidenhain's hematoxylin, the crystals stain black, and when placed in solutions of iron alum, retain their color longer than any other of the cell contents.—*F. M. Clark.*

MÜHLDORF, ANTON. **Über die Bildung und Auflösung der Wande bei der Tetradenteilung der Pollenmutterzellen von Althea rosea.** *Ber. deut. botan. Ges.*, **57**, 299–312. 1939.

In a study of the cell wall structure of *Althea rosea*, the best preparations were made from fresh anthers crushed in weak neutral red which stained the protoplasm lightly. Anthers fixed in formol or Nawaschin's formol-chromic-acid mixture and crushed with weak neutral red gave fair slides. Paraffin sections stained and mounted in balsam were very poor.—*Merritt N. Pope.*

RÄMSCH, HEINZ. **Entwicklungsformen und Degeneration im Xanthoria-Apothecium.** *Arch. Mikrob.*, **10**, 279–301. 1939.

For a microscopic study of *Xanthoria* apothecia the following technic is proposed: Place apothecia in water until swollen, and fix under a vacuum 3–20 hr. in a mixture of 0.06% osmic acid, 0.06% glacial acetic acid and 0.2% chromic acid. Wash 2–3 days in running water and run up thru alcohols from 30% to absolute. Transfer to cedar oil for at least 8 hr. in vacuum, then to celloidin of 5, 10, 30 and 50% (24 hr. for each step). Add celloidin shavings to the 50% stage until syrup is thick. Leave object in this for several days, and embed in paper tray. Harden by hanging paper tray in 85% alcohol for 24 hr. and store in 70% alcohol. Before cutting, place in 60% alcohol where a long stay improves the cutting. While 3 μ sections are easily obtainable, 6 μ to 9 μ is recommended. Stain with iron alum hematoxylin and counterstain for 5 min. with rubin S. After the iron alum, differentiate with weak grades of alcohol until the chromatin appears blue-green and the protoplasm a bright red. Good results were obtained also from Herman's safranin and gentian-violet method. Erythrosin as a single stain shows the cell membrane.—*Merritt N. Pope.*

WERGIN, W. **Uber den Aufbau pflanzlicher Zellwande. V. Mitteilung: Untersuchungen über die Baueinheiten mit Hilfe der Quellungsanalyse.** *Protoplasma*, **32**, 116–39. 1939.

After a short review of the literature (33 citations) on the microscopic structure of cellulose walls of plant cells and on methods hitherto used for causing a separation by swelling of the cellulose lamellae, the author presents his own method as follows: Cotton fibers, stretched into straight filaments by weighting their ends, are given a preliminary coating of celloidin followed by imbedding in paraffin. The sections are made as nearly as possible along the long axis of the fibers. The sections are mounted on slides without any adhesive material, the paraffin and celloidin are removed by means of alcohol-ether while the sectioned fiber is held in place by the needles of a micromanipulator. The exposed sectioned fibers are further treated by mounting in water between cover-slip and slide. The solution, run under the coverslip to induce the swelling of the cellulose lamellae, consists of 0.58 g. $Ca(OH)_2$ in 100 cc. of 25% ammonia containing a variable amount

of NaOH (about 0.4%), the concentration to be varied for regulating the swelling process.—*Robert Chambers.*

MICROÖRGANISMS

DAVIS, J. G., McCLEMONT, J., and ROGERS, H. J. **Studies in Mastitis. I. The routine diagnosis of mastitis.** *J. Dairy Research*, 10, 60–73. 1939.

Crystal violet (1:500,000) was used in Edward's medium to inhibit organisms other than that of mastitis.—*H. Macy.*

GUITTONNEAU, M. G., and BÉJAMBES, M. **Chromo-résistance et enrobage phosphocalcique des microbes chauffes dans le lait.** *Le Lait*, 19, 225–34. 1939.

Streptococcus thermophilus cells were obtained from a 16-hr. milk culture by centrifuging, resuspended in low-count raw or heated milk at the rate of 10 cells per ml., and the suspension heated for one hour at 100° C. or 20 min. at 120° F. When smears from milk which had not been heated at high temperatures before adding the cells were stained with a sat. aq. solution of methylene blue, the cells remained unstained. They were stained when the milk had been heated at high temperatures before inoculating and then reheated. The chromo-resistance was eliminated by treating the cells with dil. acetic acid or CO_2. The chromo-resistance of *S. thermophilus* acquired thru heating in fresh milk was attributed to adsorption, by the membrane of the bacterial cell, of calcium phosphates of milk liberated from the colloidal medium by heat treatment.—*H. Macy.*

HSU, C. L., and TUNG, T. **Bactericidal action of X-rays in the presence of dyes.** *Proc. Soc. Exp. Biol. & Med.*, 42, 828–30. 1939.

It is suggested that the mechanism of ordinary photosensitization and that of the lethal effect of X-rays on bacteria suspended in dye solutions may be the same, viz., oxidation. Experimental evidence was based on tests with mercurochrome, eosin, methylene blue, crystal violet and safranin O, in which were suspended various Gram-negative or Gram-positive bacteria. Exposure to X-rays was direct, with adequate controls for temperature and for possible lethal effects of dyes or X-rays as such. Bactericidal action under radiation was, in some instances, as much as 1000 times that of the dye alone. Eosin with a non-lethal quantity of H_2O_2 showed remarkable activity when irradiated either with visible rays or X-rays. Visible light apparently may be stronger or weaker than X-rays, depending on the dye and the organisms.—*M. S. Marshall.*

KLIMMER, M., and WEISKE, GERTRUD. **Zur Züchtung der Galtstreptokokken aus Milch. IV. Selektive Nährböden.** *Milchw. Forsch.*, 19, 15–22. 1937.

The authors studied, among other compounds, trypaflavin, alizarin, acid yellow, Bismarck brown, tropaeolin OO, pyronin, martius yellow, naphthol yellow, aurantia, nile blue sulfate, brilliant green, fast green, fuchsin, iodine green, crystal violet, malachite green, methyl violet 5 B, Victoria blue 4 R, in agar containing sucrose, serum, alkaline albuminate, and brom cresol purple (Klimmer, Haupt and Roots) to determine inhibition of mastitis streptococci and other organisms in milk.—*H. Macy.*

KNISELY, M. J. **A simple and time saving procedure for the identification of Treponema pallidum.** *J. Lab. & Clin. Med.*, 24, 1309. 1939.

The procedure recommended employs a colloidal aq. solution of silver sold under the name of "Collargolum", which is diluted 1:20. The technic is as follows: Place a drop of the suspected material on a slide and mix with an equal volume of the reagent. Streak out as for a blood smear. Examine microscopically when dry. The unstained *Treponema pallidum* stands out against a background of dark yellow to light brown.—*John T. Myers.*

LAWSON, GEORGE McL. **Modified technique for staining capsules of Hemophilus pertussis.** *J. Lab. & Clin. Med.*, 25, 435–8. 1940.

The writer recommends the following technic: Allow smears to air-dry; cover them with 5% aq. phosphomolybdic acid for 30 sec.; wash in water, and then in methyl alcohol. Cover with 10–20 drops of stain (Wright stain 2 parts and glycerol 1 part) for 2 min.; then add 20–30 drops of dist. water and let stand

10–20 min.; rinse, dry, and examine. Much better capsules are obtained if the culture is grown on Bordet-Gengou medium containing 5–8% of mucin.—*John T. Myers.*

McCLEMONT, J., and DAVIS, J. G. **Studies in Mastitis. IV. Mastitis in relation to the methylene blue reduction test.** *J. Dairy Research*, **10,** 88–93. 1939.

The methylene blue reduction test was not capable of detecting mastitis. Reduction time was more closely related to the cell content of the infected milk.—*H. Macy.*

MILAKNIS, ANTANAS. **Beitrag zur elektiven Züchtung des Streptococcus agalactiae und der Brucella Bang.** *Milchw. Forsch.*, **19,** 392–6. 1938.

Crystal violet in a 1:3,000,000 concentration in broth was used to determine inhibition of growth of *Streptococcus agalactiae*, a *Brucella* strain and several saprophytes. It was effective against Gram-negative bacteria and all Gram-positive rods or filamentous types, but four different *Streptococcus* strains and two *Micrococcus* strains were resistant and overgrew the mastitis strain.—*H. Macy.*

NOVEL, E. **Une technique facile et rapide de mise en évidence des cils bactériens.** *Ann. Inst. Pasteur*, **63,** 302–11. 1939.

A reliable technic is offered for staining bacterial flagella: Clean new slides in bichromate-sulfuric-acid; rinse; soak in xylene 24 hr.; then store in strong alcohol. To use, remove slide with forceps, burn off alcohol, and place on a plate at 50° C. Prepare the bacterial emulsion by removing a particle of the growth on an agar slant (avoiding water of condensation) and immerse the loop for 20–30 sec. in 2–3 cc. of water in a grease-free watch-glass. Place 3–5 drops of the emulsion separately on a slide. When drops are dry, remove slide without further fixation. Prepare fresh mordant as follows: Tannin 20%, 50 cc.; cold sat. $FeSO_4$, 40 cc.; sat. alc. solution basic fuchsin or gentian violet, 7 cc. Filter the mordant from a small paper and funnel onto the slide so as to cover all the dried areas. Allow to stand 1–1½ min. Rinse in dist. water and apply freshly prepared Fontana-Tribondeau Ag-impregnation solution ($AgNO_3$, 1 g.; water, 20 cc.; with 10% NH_4OH added to the solution drop by drop until the ppt. formed redissolves, but without going beyond point of opalescence). When preparation is light chocolate-colored (usually between 50–80 sec.) rinse in dist. water and dry on warm plate. The flagella stand out strongly, and only 3 min. are required for the whole procedure.—*S. H. Hutner.*

RITTER, CASSANDRA. **Studies of the toxicity of basic fuchsin for certain bacteria.** *Amer. J. Pub. Health*, **30,** 59–65. 1940.

Six dyes which are included in the general term basic fuchsin were tested for bacteriostatic titer against 10 strains of bacteria (coliform types, a streptococcus, and aerobic spore formers) which might be found in water. Two of the dyes were commercial basic fuchsins certified by the Biological Stain Commission: DF-4, pararosanilin acetate or a mixture of acetate and chloride, dye content 91%; and CF-15, rosanilin chloride, dye content 91%. The other four were specially prepared chloride salts of the 4 lower basic members of the magenta series, designated as follows: pararosanilin chloride (Magenta O); rosanilin chloride (Magenta I); Magenta II; and new fuchsin (Magenta III). The dyes were tested in such a way as to determine the bacteriostatic titer under the same conditions for each member of the dye series; this gave a relative titer, comparing each dye with the others, the results not being absolute for any specific dye and organism combination. The results for all organisms bore a relative titer corresponding to the number of methyl groups in the dye radical, the titer increasing with the larger number of methyl groups. The commercial samples showed bacteriostatic titers only slightly higher than those of the pure salts of which they were largely composed, suggesting the presence in these samples of small amounts of the higher homologs. Results on another series of certified commercial basic fuchsin, composed of both acetate and chloride salts, suggested that the anion has small part in determining the bacteriostatic titer. Basic fuchsin is used in fuchsin lactose broth for water analysis in a concentration of 0.0015% or 1:66,000. This concentration of pararosanilin or of rosanilin would eliminate unwanted spore-forming bacteria but not inhibit the coliform group, according

to the experiments here reported. Either rosanilin (Magenta I) or pararosanilin (Magenta O) may be used in fuschin lactose broth for bacteriostatic action, the pararosanilins being preferable. Magenta II and III are not suitable. All samples of basic fuchsin submitted for certification up to this time have been found satisfactory in accordance with the results of these experiments. Tests of new batches of dye may be made by determining the bacteriostatic titer, in comparison with the titer of the standard dye, for one coliform group organism and one aerobic spore former.—*M. W. Jennison.*

STOVALL, W. D., and BLACK, C. E. **The influence of pH on the eosin methylene blue method for demonstrating Negri bodies.** *Amer. J. Clin. Path., Tech. Suppl.*, 4, 8. 1940.

A solution of 1% ethyl eosin in 95% alcohol stained Negri bodies pale red at a reaction of pH 6.0 or more alkaline. The intensity increased at more acid reactions, reaching a maximum at pH 3.0. Loeffler's methylene blue, used as a counterstain, was most satisfactory at pH 5.3; it removed eosin at a pH-value of 6.0 or higher. The following technic is recommended:

Stain 2 min. in 1% ethyl eosin in 95% alcohol with 5.5% N/10 HCl added. Rinse in dist. water. Stain 2 min. in the following solution: sat. alc. methylene blue (medicinal), 15 cc.; dist. water, ad. 60 cc.; sodium acetate, 0.4 g.; glacial acetic acid, 0.08 cc. Rinse with dist. water. Rinse in dil. acetic acid (dist. water, 60 cc. with 13 drops of glacial acetic acid) until the color of the section changes from deep blue to brownish red. Rinse in dist. water. Run thru graded alcohols, keeping the section in abs. alcohol until the color no longer washes out, controlling the differentiation by observation under the microscope. Run into xylene, and mount in balsam. This technic gives a higher proportion of positive results. Detailed directions are given for selecting the brain tissue, preparing it, fixing, dehydrating, infiltrating in paraffin, embedding, cutting, sectioning and staining.—*George H. Chapman.*

SULLIVAN, N. P., and SEARS, H. J. **A simple technique for concentrating tubercle bacilli in sputum.** *J. Lab. & Clin. Med.*, 24, 1093–5. 1939.

Approximately 0.5 g. papain is mixed with 50 cc. sputum, and incubated at 37° C. till liquefied (usually 10–15 min.). The material is centrifuged and smears are prepared from the sediment in the usual way. The sediment may be used for animal inoculation or cultures without further treatment.—*John T. Myers.*

HISTOCHEMISTRY

BRODA, B. **Über die Verwendbarkeit von Chinalizarin, Titangelb und Azoblau zum mikro- und histochemischen Magnesiumnachweis in Pflanzengeweben.** *Mikrokosmos*, 32, 184. 1939.

A histochemical technic for demonstrating magnesium is proposed, which calls for the following reagents: (1) Quinalizarin, 1 part, triturated with 5 parts sodium acetate crystals; employed as fresh (not over 24 hr.-old) 0.5% solution in 5% aq. NaOH. (2) Titan yellow; 0.2% aq. solution. (3) Azo blue; 0.1% aq. solution. (4) NaOH; 10% aq. solution.

Technic: On each mounted paraffin section, place 1–2 drops of stain reagent 1, 2, or 3, adding 1–2 drops of 10% NaOH solution in the case of reagents 2, and 3. Put on a cover glass, and examine under a comparison microscope, with pure magnesium lake, to facilitate the detection of small quantities of magnesium. Magnesium is usually found in the globoids of aleuron granules. The typical staining appears in a few minutes. Oils interfere with the reaction and should be removed previously with alcohol-ether mixture. Quinalizarin, after several hours, produces a distinct blue stain, titan yellow stains brick red to rose, and azo blue stains violet.

The glandular cells of medicinal plants which contain magnesium give only a slightly stronger reaction than the ordinary basal cells which likewise contain magnesium. Sections of fresh stems of sugar cane are first brought into 95% alcohol before being treated with the reagents. Large irregular blue stars may be observed in the interstitial spaces. *Panicum virgatum* (millet) serves as test material for determination of magnesium. (Leaves, stems, roots, and shoots may be used.) Quinalizarin in strong ammoniacal solution may be used as a reagent for the detection of calcium oxalate (e.g., in stems of rhubarb, *Rheum rhaponticum*).—*J. M. Thuringer.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the January number of this Journal.

STAINS CERTIFIED DEC. 1, 1939 TO FEB. 29, 1940*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Giemsa stain	LGe–1	....	As blood stain	Dec. 4, 1939
Thionin	NT–6	85%	As histological stain and for frozen tissue	Dec. 7, 1939
Basic fuchsin	LF–5	94%	For general staining, the Feulgen reaction, and in bacteriological media	Dec. 26, 1939
Cresyl violet	NW–8	86%	For use in histology	Jan. 16, 1940
Wright stain	LWr–9	....	As blood stain	Jan. 16, 1940
Toluidine blue	NU–3	59%	For general histological staining	Jan. 8, 1940
Methyl green	NG–14	78%	In histology and as constituent of Pappenheim stain	Jan. 19, 1940
Resazurin	NRz–1	....	For testing reduction in milk	Jan. 23, 1940
Wright stain	NWr–14	....	As blood stain	Feb. 7, 1940

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

STAIN TECHNOLOGY

VOLUME 15 JULY, 1940 NUMBER 3

AN OSMIC IMPREGNATION METHOD FOR MITOCHONDRIA IN PLANT CELLS

EARL H. NEWCOMER, *Michigan State College*[1], *East Lansing, Mich.*

Osmium tetroxide, in technics like Kolatchev's (1927–8) and its various modifications, has been used for years in animal cytology for the preservation of various protoplasmic constituents, such as the Golgi apparatus. Bowen (1927–8), using the Kolatchev technic on plant tissues, described a new category of cytoplasmic inclusions

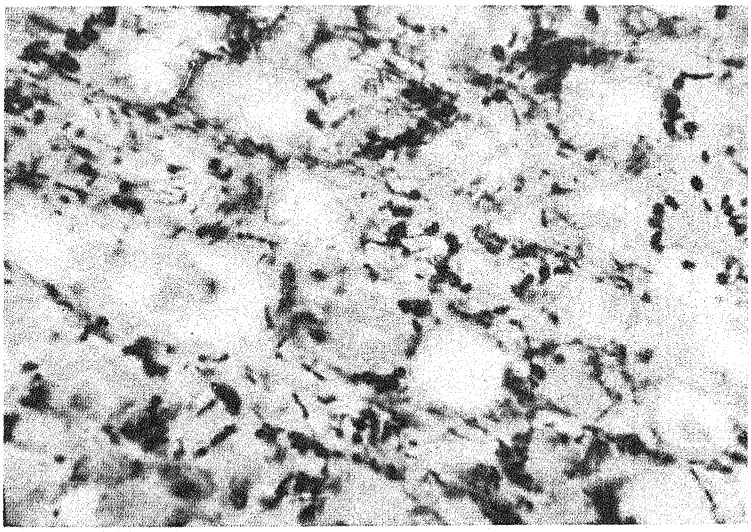

Fig. 1. Photomicrograph of root-tip cells of *Pisum*, × 1425. The preparation is unstained.

which he called the "osmiophilic platelets." The author (1940), in duplicating some of Bowen's work, used this technic and observed the bodies described by Bowen which he considered to be deformed chondriomes caused largely by the initial fixation. A simple modification of the technic, however, in which Zirkle's fluid was substituted for Champy's, resulted in preparations in which the chondriome and

[1]Journal article No. 362, n.s.

proplastids were beautifully preserved and selectively blackened by the osmium tetroxide, showing clearly the transition stages between the two categories. No staining is necessary after bleaching, the chondriome and proplastids appearing jet black against the light gray background of cytoplasm and nuclei (Fig. 1). The nuclei, cytoplasm and cell walls can be stained with Altmann's mixture of acid fuchsin and anilin oil (Lee, 1937, p. 305) followed by a counterstain of gold orange in clove oil. The result is a brilliant triple stain, with the nuclei red, cytoplasm and cell walls orange, and the chondriome black.

A summary of the technic is as follows:

1. Cut root-tips or other material directly into Zirkle's[2] fixative and fix for 48 hours.
2. Wash 8 hours or over night in running tap water.
3. Treat with 2% OsO_4 for 4–6 days—change solution on alternate days. (It was found impossible to standardize this step of the technic, because root-tips in the same bottle will exhibit different rates of reduction of the OsO_4.)
4. Wash 8 hours or over night in tap or distilled water.
5. Dehydrate by the usual alcoholic series substituting benzene for xylene in clearing, or by the shorter *N*-butyl-alcohol–ethyl-alcohol series of Lang (1937).
6. Embed in 52° paraffin and section at 5 μ.
7. Bleach as follows: 1% $KMnO_4$, 5 minutes. Rinse in distilled water, then place in 3% oxalic acid for 2–3 minutes. Wash in running tap water for 15 minutes.
8. Dehydrate thru graded alcohols, clear in xylol and mount in balsam. Or, if the preparation is to be stained, pass from water into acid fuchsin (pipette acid fuchsin on slide, heat until steaming, let stand for 5 minutes, repeating the procedure once more, and when cool, wash in water), and ascend thru the alcohols, counterstaining in 1% gold orange in clove oil after absolute alcohol. After the counterstain, wash and clear in xylol and mount in balsam.

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Lang, A. G. 1937. The use of *N*-butyl alcohol in the paraffin method. Stain Techn. 12, 113–7.

Lee, B. 1937. The Microtomist's Vade-Mecum, *10th Ed.*, edited by J. Brontë Gatenby and T. S. Painter. Churchill, London.

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[2]Zirkle's fixative:

3% $K_2Cr_2O_7$		1.25 g.
3% $(NH_4)_2Cr_2O_7$		1.25 g.
3% $CuSO_4$		1.00 g.
Distilled water		100.00 g.

THE DETERMINATION OF APPARENT ISOELECTRIC POINTS OF CELL STRUCTURES BY STAINING AT CONTROLLED REACTIONS[1]

NORMAN D. LEVINE, *Animal Pathology Laboratory, University of Illinois, Urbana, Ill.*

ABSTRACT.—The staining reactions at controlled pH-values of various dyes with the nucleus and cytoplasm of *Trichonympha collaris* under different conditions were investigated. When staining intensity was plotted against pH, it was found that with each dye a different curve was obtained. "Isoelectric points" obtained by superposition of acid and basic dye curves varied for the same material with the dyes employed. It was found that, with the same dye, the curves of staining intensity plotted against pH varied with the buffer system utilized. Moreover, the intensity of staining at any pH was found to vary directly with the concentration of dye and inversely with the concentration of buffer. Various factors modifying staining intensity were studied. In the staining of a protein in buffered solution, it was shown that staining intensity (the index of the concentration of the dye-protein compound) at a given pH-value is dependent upon the interaction of the dye-protein, buffer-protein and dye-buffer systems, and that as the dye or buffer or their concentrations were varied, the resultant "isoelectric points" which were obtained also varied. In view of these facts and of the present lack of knowledge of dyes and dye-protein combinations it would be impossible to determine a true isoelectric point by staining at controlled pH-values without further extensive work on the subject. It follows that no true isoelectric points have hitherto been obtained for nucleus, cytoplasm or other tissue elements by staining at controlled pH.

INTRODUCTION

Since Loeb (1922) showed that it was possible to approximate the isoelectric point of gelatin by staining it with certain acid and basic dyes in a series of buffered solutions, a number of workers have applied this method to determine the "isoelectric points" of the nucleus and cytoplasm of various tissues.

The theory underlying this method of determining isoelectric

[1]From a dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Zoology in the Graduate Division of the University of California.

points is as follows: If the amount of dissociation of a protein or other ampholyte is plotted against pH, a curve will be obtained having two maxima and one minimum. The pH at which the minimum occurs is defined as the isoelectric point of the protein. On the more acid side of the isoelectric point (i.e., at lower pH-values), the protein is dissociated as a positively charged ion. On the other side of the isoelectric point, it is dissociated as a negatively charged ion. Near the isoelectric point, however, there will be a certain small percentage of negative ions on the acid side, and a similar small percentage of positive ions on the basic side, since the dissociation curves of the acid and basic groups of the protein are sigmoid in shape, and the isoelectric point is the point at which the two cross.

Acid dyes dissociate to form negatively charged dye ions. Basic dyes dissociate into positively charged dye ions. Therefore the acid dyes will combine with the positively charged protein ions at pH-values below the isoelectric point, and basic dyes will combine with the negatively charged protein ions at pH-values above the isoelectric point. Assuming the acid dye to be completely dissociated, or nearly so, if a series of dye solutions is set up at different pH-values and samples of a protein are stained in them, the intensity of staining will depend on the amount of ionization of the protein. Hence if intensity of staining is plotted against pH, a sigmoid curve should result, which indicates the amount of dissociation into positive ions of the protein. Similarly, with a basic dye a curve is obtained which indicates the amount of dissociation of the protein into negative ions. The isoelectric point should be the point at which these two curves cross. It will be noted that the above discussion is based on the stoichiometric theory of staining. This is done merely for the sake of convenience, as the adsorptive theory would be equally applicable. For a more detailed discussion than is given here, the paper of Craig and Wilson (1937) may be consulted.

Previous workers, staining tissues as outlined above, have found that with acid dyes the intensity of staining decreases as the pH increases, that with basic dyes the intensity increases with increasing pH, and that the curves obtained are roughly sigmoid. With this confirmation of the theory, they have proceeded to determine isoelectric points for the nucleus and cytoplasm of a wide variety of tissues. None of them, however, has attempted a systematic investigation of the effects of various factors on the isoelectric points obtained. In view of the number of papers on the subject which have been published, and of the significance of the results if valid, it was determined to subject the method itself to a critical evaluation. Fac-

tors which, it was considered, might be important were (1) the dye used, (2) the concentration of dye used, (3) the buffer salts used, and (4) the concentration of buffer used. Each of these was varied independently of the other three, and the results of such treatment noted.

Historical

Pischinger (1926) made the first attempt to determine the isoelectric points of tissue elements. Practically all later workers used his method or a modification of it. He stained gelatin, egg albumin, and alcohol-fixed thymus and cartilage with toluidine blue (a basic dye) and cyanol extra (an acid dye) at a series of pH-values, and plotted the logarithm of the amount of stain against pH. The amount of dye taken up was determined by extracting after staining, and using a colorimeter.

He also stained sections on slides of alcohol-fixed tissues. The slides were stained ten minutes and washed briefly with the proper buffers. The toluidine blue preparations were placed in 4% ammonium molybdate to prevent extraction of stain in alcohol. Most of the water was removed with filter paper, and the slides passed thru xylene-alcohol and xylene to balsam. Stain intensity was estimated by microscopic inspection. Pischinger gives a table of isoelectric points and ranges for the nucleus, cytoplasm and tissue elements of various tissues. These differ for different tissues.

In one case information is given on the effect of buffer concentration on the staining. With toluidine blue, Pischinger found that the neurofibrils stained more intensely and in a more acid region in M/20 buffers than in M/200 buffers. The stain intensity of the former at pH 5.2 was about equal to that of the latter at pH 6.0.

Methyl violet 6B, safranin, Congo red, and picric acid were also used. Pischinger states that the results with safranin and picric acid were in general the same as those for toluidine blue and cyanol extra respectively. With methyl violet, the isoelectric point was shifted toward the acid side. With Congo red, there was a slight decrease in staining on the alkaline side, but even at pH 7.6 (the highest used) the tissues contained a considerable amount of dye. At no pH-value were the nuclei stained, but the dye was precipitated below pH 3.3, so Pischinger could go no lower.

In spite of these contradictory results, Pischinger believed that he had obtained true isoelectric points with toluidine blue and cyanol extra. In a later paper (1927) on the isoelectric points of muscle constituents, he changed his dyes to methylene blue and crystal ponceau.

Other investigators were Pulcher (1927), who worked with smears of frog blood, guinea pig blood, egg albumen, fresh fibrin and "stromi di emazie"; Mommsen (1927), Schwartz-Karsten (1927) and Ochs (1928), who worked with air-dried human blood streaks; Tolstoouhov (1927, 1928), working with blood smears and tissue sections; and Pfeiffer (1929), who worked with plant tissues.

Zieger (1930a, b) continued Pischinger's work by investigating the effect of the fixing fluid on the isoelectric points of a number of tissues. He used M/200 methylene blue and crystal ponceau as his basic and acid dyes. Each fixative gave a different isoelectric point for the same tissue, but with different tissues the shift in isoelectric point as compared with that of absolute alcohol was not invariably in the same direction. Altho, as this paper will show, these so-called "isoelectric points" obtained by Zeiger are not the actual isoelectric points of the tissues, nevertheless his paper is of interest. The relative shifts in isoelectric range are possibly valid.

Others who used similar methods to obtain isoelectric points of various tissues were Yasuzumi (1933a, 1933b, 1934), Nishimura (1934), Yasuzumi and Matsumoto (1936), Sturm (1935), Achard (1935), Fautrez (1936) and Ikeda (1935, 1936).

A lengthier historical account is given by Levine (1937).

Method

The material selected for the investigation was the hypermastigote flagellate, *Trichonympha collaris.* This protozoon is to be found in great abundance in the intestine of the damp-wood termite *Zootermopsis angusticollis*, which is common in the San Francisco Bay area. Thousands of organisms are present in one termite. Coverslip smears were made by removing the hindgut, breaking its wall, and distributing the exuding material as evenly as possible on the coverslip with a pair of dissecting needles. In order to cleanse their intestines of wood particles, whose color would obscure the staining reactions, the termites were previously fed on filter paper for at least a week before use.

Before any drying could take place the smears were dropped into a fixative. Five different fixatives were used, Susa's, Schaudinn's, Bouin's, absolute alcohol, and liquid air. The first three fixatives were used at 60° C., the absolute alcohol at room temperature. Fixation lasted 30 minutes except with liquid air, in which an immersion of 30 seconds was sufficient. Material fixed in liquid air could not be stained at pH-values above 5.0, because the low acidity allowed the protozoa to come off the coverslips. Liquid air fixation

was also unsatisfactory with acid dyes. The protozoa stained readily, but the stain came out quickly in the subsequent washing process. Therefore no isoelectric points could be calculated for this fixative. With this exception, all fixatives were used in all stains.

After fixation the coverslips were washed in running tap water for an hour to remove the fixatives, and then transferred to the staining solutions. After staining for 36 hours, at which time equilibrium was very nearly approached, the smears were washed thoroly in distilled water. Then the coverslips were passed thru four changes of tertiary butyl alcohol and two changes of neutral xylene, and mounted in neutral Canada balsam. The tertiary butyl alcohol prevented the loss of stain which would have taken place in ethyl alcohol. (Levine, 1939).

All staining solutions were prepared in the same way. Dye and buffer solutions of twice the concentration required were made up, and equal quantities of each were mixed to obtain the final staining solutions. The actual pH-values of the solutions were determined by means of a glass electrode. The addition of dye changed the pH-values of the buffers, the amount of change depending on the dye and on its concentration. In order to obtain buffer or dye solutions of different concentrations, the concentrations of the stock solutions were varied, or the buffered solutions were diluted as required.

The following series were run:

1. The effect of different dyes on the "isoelectric point" obtained.

Fixatives:—Susa's, Schaudinn's, Bouin's, absolute alcohol, liquid air.

Buffer:—McIlvaine's citric-acid–secondary-sodium-phosphate buffers diluted ten times (referred to hereinafter as 1/10 McIlvaine's buffers), at approximate pH values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0.

Dyes:—Basic dyes—0.0001 M[2] toluidine blue O (National Aniline Co., Cert. No. NU-2, dye content 60%), 0.0001 M methylene blue U. S. P. medicinal (National Aniline Co., Cert. No. NA-48, dye content 89%), 0.0001 M Nile blue sulfate (National Aniline Co., Cert. No. NNb-1, dye content not given), and 0.0001 M crystal violet (A. H. Thomas Co., no certification number or dye content given).

Acid dyes—S/20[3] ponceau 2R (Coleman and Bell Co., no certification number or dye content given), and 0.0001 M orange G (National Aniline Co., Cert. No. NO-3, dye content 85%).

[2]The concentrations of dye solutions were made on the basis of the dye contents of the dry dyes, except in the cases of Nile blue sulfate, crystal violet and ponceau 2R, for which dye content was not known. In these cases it was arbitrarily assumed to be 80%.

[3]When a 0.002 M stock solution of ponceau 2R was made up, it was found that the dye did not all dissolve even after several days. This solution was then filtered, and the filtrate considered a saturated solution. The symbol S/20 thus indicates a 1/20 saturated solution.

2. The effect of concentration of dye on the isoelectric point obtained.

Fixatives:—As above.
Buffer:—As above.
Dyes:—The same six dyes were used as above, in concentrations of 0.0001 *M*, 0.00025 *M*, 0.0005 *M*, and 0.001 *M*. In the case of ponceau 2R the concentrations were S/20, S/8, S/4, and S/2.

3. The effect of different buffers on the isoelectric point obtained.

Fixatives:—As above.
Buffers:—All buffers were diluted to one-tenth their standard strength.
(1) 1/10 McIlvaine's buffers (citric acid and secondary sodium phosphate) at pH-values approximately 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0.
(2) 1/10 Sörenson's buffers (sodium citrate and HCl) at pH-values approximately 1.0, 2.0, 3.0, 4.0 and 5.0.
(3) 1/10 Sörensen's buffers (primary potassium phosphate and secondary sodium phosphate) at pH-values approximately 5.0, 6.0, 7.0 and 8.0.
(4) 1/10 Clark and Lubs' buffers (potassium acid phthalate and HCl) at pH-values approximately 3.0 and 4.0.
(5) 1/10 Clark and Lubs' buffers (potassium acid phthalate and NaOH) at pH-values approximately 5.0, 6.0 and 7.0.
Dyes:—(1) 0.0001 *M* toluidine blue O.
(2) S/20 ponceau 2R.

4. The effect of different concentrations of buffer on the isoelectric point obtained.

Fixatives: As above.
Buffers:—The same buffers were used as in the preceding series. McIlvaine's and Sorensen's citrate-HCl buffers were used in standard, 1/10 standa d, and 1/100 standard concentrations, the other buffers in standard and 1/10 standard concentrations.
Dyes:—(1) 0.0001 *M* toluidine blue O.
(2) S/20 ponceau 2R.

To determine intensity of staining, color charts were prepared as follows: A series of eight small squares was ruled off on white drawing paper. The first square was painted evenly with a 1% solution of one of the dyes, the second square with a 0.5% solution, the third with a 0.25% solution, and so on down to the last square, which was faintly colored by the 1/128% dye solution. Thus a geometric progression in intensity was obtained, each step being double the intensity of the preceding one. These charts had approximately the same shade of color as the slides, except in the case of crystal violet, in which the stained organism contained more red than did the color chart. The difference was not sufficient to interfere with color comparison, however. With the aid of these charts, the intensity of staining of the *Trichonympha* nucleus and cytoplasm under the various experimental conditions was recorded. The methylene blue chart was used on

organisms stained in Nile blue sulfate and on the nuclei stained in toluidine blue O, since these dyes gave its color and not those of their own charts. While this method involves the use of the eye in comparing intensities, it was found that after some practice a high degree of accuracy was obtained as judged by the results of repeated determinations made on the same material at different times.

RESULTS

The experimental values obtained were plotted on graph paper with stain intensity on the ordinate, and pH on the abscissa. From these graphs, "isoelectric points" were obtained and tabulated. In

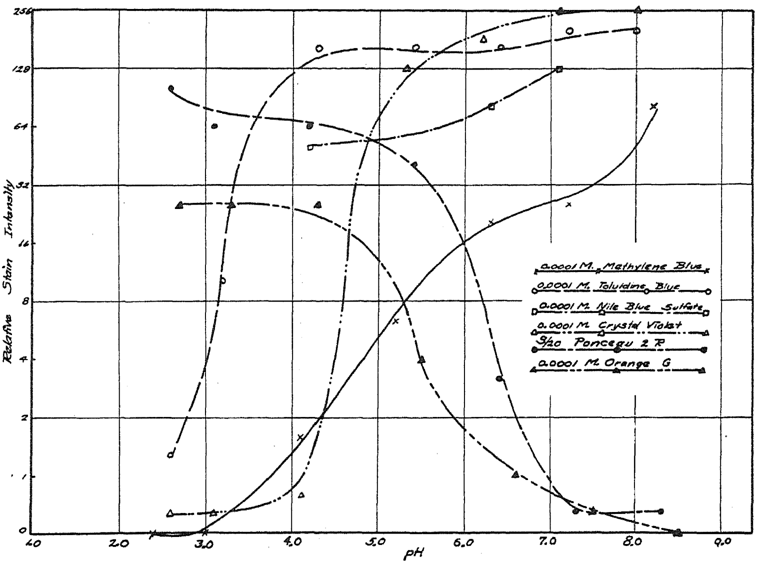

Fig. 1. The effect of different dyes on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1–10.

all, 136 graphs were constructed and used, but it is obviously impracticable to present more than a few representative ones in this paper. All the other graphs are to be found in the writer's doctorate thesis (Levine, 1937).

SERIES 1. THE EFFECT OF DIFFERENT DYES ON THE "ISOELECTRIC POINT" OBTAINED.

In Fig. 1 stain intensity is plotted against pH for the six dyes used with *Trichonympha collaris* nucleus after Susa's fixation.

Theoretically, if the curves determine isoelectric point, those of the four basic dyes should all be the same curve or approximately the

same, and those of the two acid dyes should be the same. The pH-value at which these two ideal curves crossed would be the isoelectric point of the cell element. This is obviously far from the fact.

Table 1 gives the "isoelectric points" obtained from the intersection of the acid and basic curves for the nucleus of *Trichonympha collaris.* With any given fixative, the isoelectric point should be the same regardless of the dyes used in obtaining it. Therefore all the figures in each vertical column of each table should be the same. Far from this, Table 1 gives values for the nucleus fixed in Susa's ranging from 3.3 to 6.0, with Schaudinn's from less than 2.6 to 4.9, with Bouin's from 3.8 to 5.6, and with absolute alcohol from much less than 2.6 to 4.8. Similar results were obtained for the cytoplasm.

TABLE 1. "ISOELECTRIC POINTS" OF TRICHONYMPHA COLLARIS NUCLEUS

Dye Combination*	Fixative			
	Susa's	Schaudinn's	Bouin's	100% Alcohol
P—TB	3.6	3.5	4.7	3.3
OG—TB	3.3	3.0	4.2	3.0
P—CV	4.9	3.0	4.7	<<2.6*
OG—CV	4.7	2.9	4.5	<<2.6
P—NBS	4.9	3.1	4.7	<2.6
OG—NBS	<4.2	<2.6	3.8	<2.6
P—MB	6.0	4.9	5.6	4.8
OG—MB	5.3	3.9	5.3	4.3

*The symbols used in the table are:
P = Ponceau 2R
OG = Orange G
TB = Toluidine blue O
CV = Crystal violet
NBS = Nile blue sulfate
MB = Methylene blue
<< = "much less than"

Even if the results with only one acid dye are used, the range becomes only a little narrower. For instance with ponceau 2R as the acid dye, the isoelectric points of the nucleus range with Susa's fixation from 3.6 to 6.0, with Schaudinn's from 3.0 to 4.9, with Bouin's from 4.7 to 5.6, with absolute alcohol from much less than 2.6 to 4.8. Similar results are obtained with the cytoplasm, or with orange G substituted for ponceau. With this assortment of "isoelectric points," it would seem difficult to pick the correct one, if any is correct.

Certain facts appear from the data. First, orange G gives a consistently lower "isoelectric point" with any of the basic dyes than does ponceau 2R. Second, methylene blue gives a consistently higher "isoelectric point" with either of the acid dyes than does any other basic dye. The other three basic dyes do not arrange them-

selves in any consistent consecutive order. Third, when the fixation is the same, the "isoelectric points" of the cytoplasm are consistently higher than those of the nucleus. This is in accord with the results of Pischinger (1926, 1927), Zeiger (1930 a, b), Craig, and Wilson (1937), etc.

SERIES 2. THE EFFECT OF CONCENTRATION OF DYE ON THE ISOELECTRIC POINT OBTAINED.

In Fig. 2 stain intensity is plotted against pH for different concentrations of the same dye. The results obtained on *Trichonympha collaris* nucleus fixed in Bouin's and stained with toluidine blue O are shown. The curves obtained with the other combinations of dyes and fixatives are essentially similar in their variability.

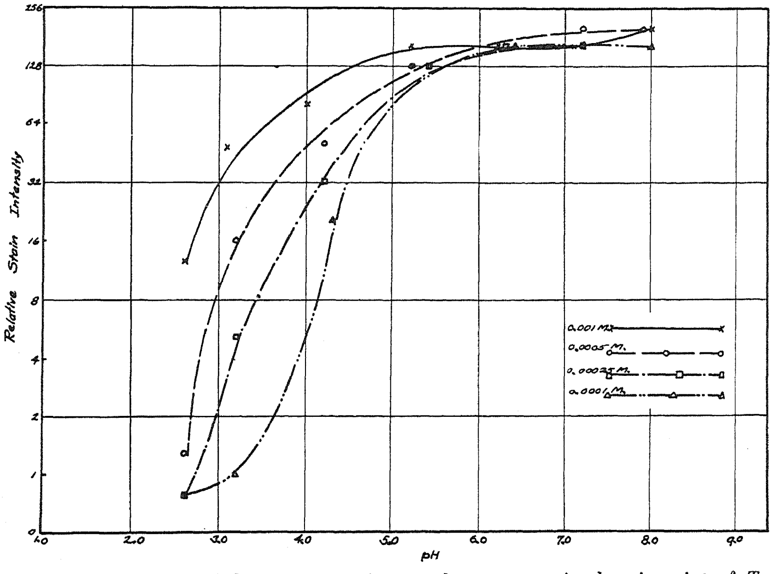

Fig. 2. The effect of dye concentration on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Bouin's. Dye—toluidine blue O. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1–10.

An analysis of these graphs shows that different concentrations of dye give different curves, which may be more or less widely separated. Usually, the higher the concentration of basic dye, the further to the acid side is the resultant curve, and the lower would be the "isoelectric point" obtained by its use. With acid dyes, the higher the concentration of dye, the further to the basic side is the resultant curve, and the higher would be the "isoelectric point" obtained; this point is illustrated in Fig. 3. These two opposite tendencies cancel each other out in part, but there is still a great discrepancy in the

resultant isoelectric points. In Table 1 are given the "isoelectric points" obtained for Schaudinn-fixed *Trichonympha* nucleus by superposition of the acid and basic dye curves. The degree of variability of "isoelectric points" of nucleus and cytoplasm with the other fixatives was essentially similar.

It is apparent that as the concentration of acid dye decreases, the pH of the "isoelectric point" also tends to decrease, and as the concentration of basic dye decreases, the pH of the "isoelectric point" tends to increase. This general rule has many exceptions, of which a large number are due to the positions of the points of intersection of

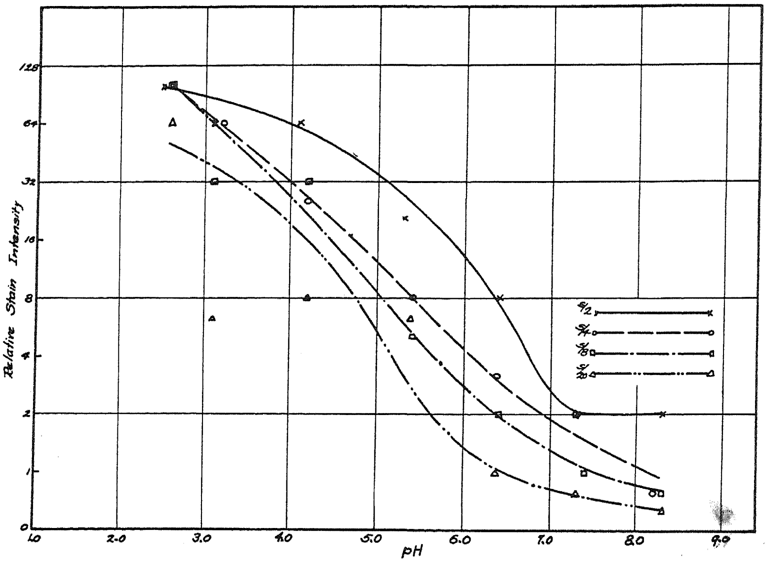

Fig. 3. The effect of dye concentration on the apparent isoelectric point of *Trichonympha collaris* cytoplasm. Fixative—Schaudinn's. Dye—ponceau 2R; dilution symbols (S/2, S/4, etc.), indicate degree of saturation. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1–10.

the acid and basic curves. If a true isoelectric point were being obtained, the two curves should intersect near their lower ends, for the isoelectric point represents a minimum amount of dissociation. But this is seldom the case. Most intersections occur near the upper extremities of one or both curves, some near the midpoint, and few below the midpoint. This is another point indicative that the true isoelectric point is not obtained.

SERIES 3. THE EFFECT OF DIFFERENT BUFFERS ON THE ISOELECTRIC POINT OBTAINED.

In figure 4 are shown the curves obtained with 0.0001 *M* toluidine blue O on Susa-fixed *Trichonympha collaris* nucleus. The results

TABLE 2. THE EFFECT OF DYE CONCENTRATION ON THE "ISOELECTRIC POINT" OF SCHAUDINN-FIXED TRICHONYMPHA COLLARIS NUCLEUS

Acid Dye	Basic Dye							
	Crystal Violet				Toluidine Blue O			
	0.001 *M*	0.0005 *M*	0.00025 *M*	0.0001 *M*	0.001 *M*	0.0005 *M*	0.00025 *M*	0.0001 *M*
Ponceau 2R								
S/2*	2.3	2.5	2.6	3.4	3.0	3.3	3.6	3.9
S/4*	2.6	2.7	2.7	3.3	3.0	3.2	3.5	3.8
S/8*	2.4	2.6	2.6	3.3	3.0	3.2	3.5	3.8
S/20*	<2.4	<2.5	<2.6	3.1	2.8	2.9	3.3	3.6
Orange G								
0.001 *M*	<2.4	<2.5	<2.6	2.7	<2.6	<2.6	2.6	3.2
0.0005 *M*	<2.4	<2.5	<2.6	2.8	<2.6	<2.6	2.6	3.3
0.00025 *M*	<2.4	<2.5	<2.6	2.8	<2.6	<2.6	2.8	3.3
0.0001 *M*	<2.4	<2.5	<2.6	2.7	<2.6	<2.6	<2.6	3.0

Acid Dye	Basic Dye							
	Nile Blue Sulfate				Methylene Blue			
	0.001 *M*	0.0005 *M*	0.00025 *M*	0.0001 *M*	0.001 *M*	0.0005 *M*	0.00025 *M*	0.0001 *M*
Ponceau 2R								
S/2*	2.8	3.4	3.5	3.7	5.1	5.0	5.2	5.7
S/4*	2.6	3.3	3.4	3.6	4.9	4.8	5.0	5.4
S/8*	2.6	3.3	3.4	3.6	4.9	4.7	4.9	5.3
S/20*	<2.4	3.0	3.1	3.4	4.8	4.5	4.7	5.0
Orange G								
0.001 *M*	<2.4	<2.5	<2.6	<2.6	3.1	3.7	4.1	4.5
0.0005 *M*	<2.4	<2.5	<2.6	<2.6	3.4	3.9	4.2	4.6
0.00025 *M*	<2.4	<2.5	<2.6	<2.6	3.7	4.0	4.2	4.6
0.0001 *M*	<2.4	<2.5	<2.6	<2.6	2.6	2.9	3.5	3.8

*S/2 = half saturated solution, S/4 = quarter saturated solution, etc.

obtained with the other fixatives and with ponceau 2R are similar. It is seen that at a given pH the same stain intensity is not obtained with different buffers. Table 3 gives the "isoelectric points" of *Trichonympha* nucleus under different conditions of fixation and buffering. The isoelectric points were determined by superimposing the curves for ponceau 2R on those for toluidine blue O. With the nucleus, the range of "isoelectric points" obtained with the same fixative covers from 0.9 pH-units (Bouin's) to 1.7 pH-units (Susa's). A similar variation was observed with the cytoplasm. The buffers can be arranged in a regular series with regard to their effect on the

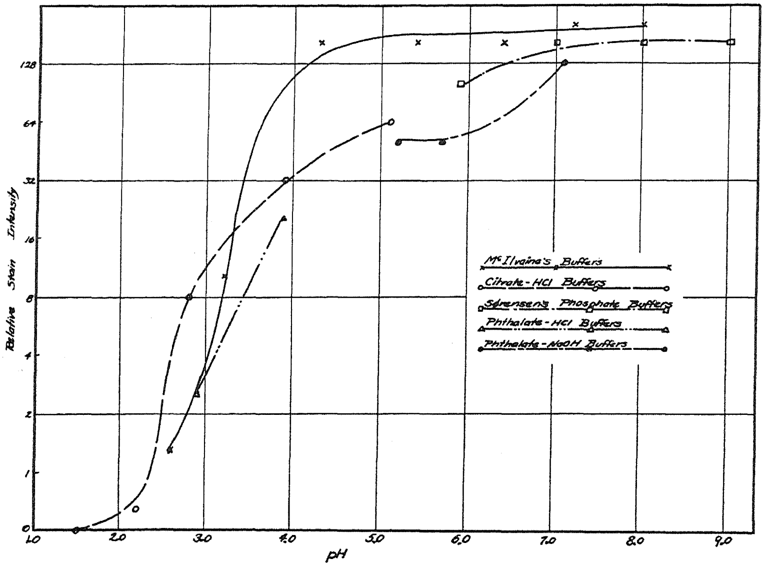

Fig. 4. The effect of different buffers on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. Dye—toluidine blue O. All buffers 1/10 standard concentration.

"isoelectric point." McIlvaine's buffers (citric acid and secondary sodium phosphate) give the lowest isoelectric points; Sōrensen's buffers (sodium citrate and HCl) give higher isoelectric points; and Clark and Lubs' buffers (potassium acid phthalate and NaOH) give still higher ones. If the other two buffers had covered a wide enough pH range, they would undoubtedly have given still different "isoelectric points."

SERIES 4. THE EFFECT OF THE CONCENTRATION OF BUFFER ON THE ISOELECTRIC POINT OBTAINED.

Figure 5 shows the effect of the concentration of McIlvaine's buffer on the isoelectric point of *Trichonympha* nucleus, when

stained with toluidine blue O after fixation in Susa's. A similar variability was obtained with the other fixatives, with ponceau 2R and with the other buffers.

TABLE 3. THE EFFECT OF DIFFERENT BUFFERS ON THE "ISOELECTRIC POINT" OF TRICHONYMPHA COLLARIS NUCLEUS

Fixative	Buffer		
	McIlvaine's	Citrate-HCl	Phthalate-NaOH
Susa's	3.7	4.6	5.4
Schaudinn's	3.6	3.9	<5.2
Bouin's	4.7	4.5	5.6
Alcohol	3.4	3.7	<5.2

The accuracy of the very dilute buffer curves (i.e., 1/100 the original concentration) is doubtful. It is very probable that in such dilute systems the reactions changed in the course of the staining process. If these are omitted from consideration, it is seen that as a general

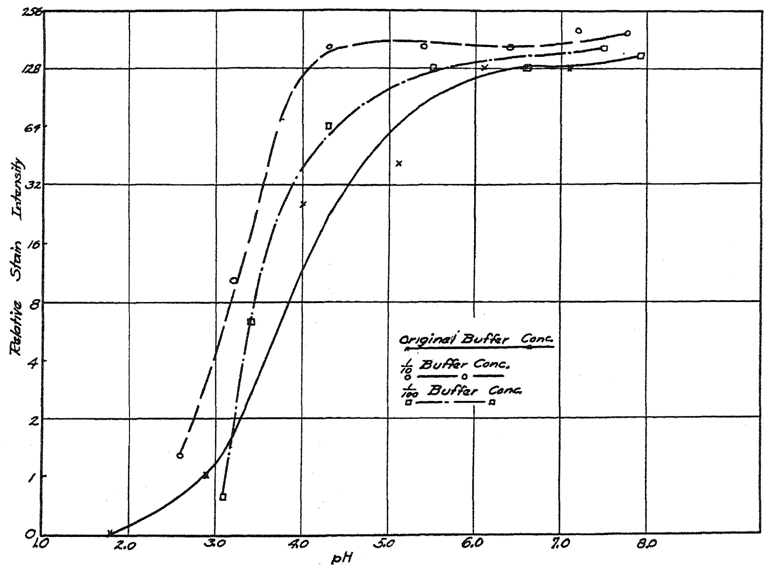

Fig. 5. The effect of buffer concentration on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. Dye—toluidine blue O. McIlvaine's buffers (citric acid and secondary sodium phosphate).

rule the more concentrated the buffer, the less intense is the staining at any given pH-value.

Table 4 gives for *Trichonympha* nucleus the isoelectric points obtained from the graphs by superimposing the ponceau curves on the toluidine blue ones. The lowered staining intensity with increased buffer concentration would tend to produce a higher iso-

electric point with toluidine blue and a lower one with ponceau. These two opposite effects partially cancel each other out, but as a result the isoelectric points obtained have no constant relation to each other.

STAINING OF CASEIN

In order to check the experiments on *Trichonympha*, casein was stained at different pH-values with toluidine blue O and methylene blue. The casein, prepared by the method of Van Slyke and Baker and washed thirty days, was obtained from Dr. P. L. Kirk of the Department of Biochemistry, University of California. One-tenth-gram samples were placed in 10 cc. of 0.0003 M dye made up in 1/10 McIlvaine's buffers (citric acid and secondary sodium phosphate) at pH values approximately 2.2, 3, 4, 5, 6, 7, and 8. The relative amounts of stain taken out of solution by the protein at different reactions was noted after 24, 48, and 72 hours. There was no essential difference between the results at these times.

TABLE 4. EFFECT OF BUFFER CONCENTRATION ON THE "ISOELECTRIC POINT" OF TRICHONYMPHA COLLARIS NUCLEUS

Buffer	Fixative			
	Susa's	Schaudinn's	Bouin's	Alcohol
McIlvaine's				
Standard Conc.	4.4	3.3	4.3	2.8
1/10 Conc.	3.7	3.4	4.3	3.3
1/100 Conc.	3.7	3.7	4.2	3.8
Citrate-HCl				
Standard Conc.	>4.7	3.6	>4.7	3.1
1/10 Conc.	4.7	3.9	4.6	3.7
1/100 Conc.	4.7	3.8	4.4	3.5
Phthalate-NaOH				
Standard Conc.	6.5	5.7	5.9	<5.0
1/10 Conc.	5.4	<5.2	5.6	<5.2

At pH 6, some protein was dissolved; at pH 7 most of it was dissolved, and at pH 8 all the casein went into solution. These three pH-values were therefore valueless, since it was impossible to determine how much dye was combined with the protein, and how much was not. At any lower pH-value, however, it was found that relatively more methylene blue than toluidine blue was present in the supernatant fluid above the dye-protein precipitate. Therefore methylene-blue-casein is more completely ionized at these reactions than is toluidine-blue-casein. Hence the intensity of staining of casein is less with methylene blue than with toluidine blue. This result is in complete accord with the results obtained for *Trichonympha*.

Discussion

In order to apply a staining method to the determination of isoelectric point, the cells must be killed and fixed. Unless the cell is dead, most dyes will not penetrate its outer membrane. Therefore in no case does the method establish the isoelectric points of the living proteins, but only those of the proteins of the dead organism. Different fixatives, as Zeiger (1930b) has shown, give different isoelectric points. It has been assumed that absolute alcohol fixation does not alter the isoelectric points of tissue proteins. It is possible, however, that alcohol does in fact shift the isoelectric point. Certainly it changes the nature of the proteins markedly.

Except in the case of blood cells, it has been necessary to section the tissues before they could be stained. Before sectioning, the fixed block of tissue must first be passed thru increasing grades of alcohol, xylene or toluene, and paraffin. Then, after mounting, the sections must again be passed thru xylene and alcohol to water before staining. This treatment undoubtedly has some effect on the proteins. The resultant effect is not that of the fixative alone. The effect of a post-fixation in alcohol, xylene, paraffin, etc., is added. The degree to which this may be a disturbing factor is not known. In the present investigation the necessity of sectioning was eliminated by the use of protozoan smears.

In all work reported on blood streaks, the streaks are air-dried before staining. Air-drying, as Seki (1934) has pointed out, makes the cell membranes more impermeable to staining solutions, and also has the more serious effect of markedly altering the "isoelectric points" of the cell proteins. The experiments in which this took place are, however, of value with respect to the development of a practical, pH-controlled blood stain.

Most of the investigations of "isoelectric point" have utilized concentrated dye solutions and a short staining period. A ten-minute immersion was the usual time, and equilibrium was not reached. This treatment introduces another unknown variable into the reaction, i.e., the rate of staining. Its elimination would be desirable. This can easily be accomplished by using dilute staining solutions, and allowing the reaction to proceed to equilibrium, as was done in the present work.

When the slides are run up thru alcohol before mounting, there is always a loss of stain in the alcohol. This is particularly serious in the case of methylene blue and toluidine blue, the basic dyes used by most investigators. Such a loss of stain would result in throwing the results far off. In order to prevent it as much as possible, slides

stained in these two dyes were fixed by these investigators in ammonium molybdate solution before being passed into alcohol. Altho this treatment is helpful, it nevertheless allows a great deal of dye to escape. For this reason, results previously reported with these two dyes should be viewed with some suspicion. Levine (1939), however, found that the substitution of tertiary butyl alcohol for ethyl alcohol in the dehydration process eliminated practically all loss of stain. This technic was used in the present work, and ammonium molybdate fixation was not found to be necessary.

To obtain the isoelectric point of a protein from its staining properties, the intensity of staining with an acid and a basic dye should be plotted against pH. Two opposite curves will be obtained, and the isoelectric point is theoretically the pH-value at which the curves cross. This is the basis on which Yasuzumi obtained his isoelectric points. Pischinger, Zeiger, etc., however, used only the curves for their basic dyes, and considered the isoelectric points to be the midpoints of these curves. This assumption is not valid.

Many investigators have not determined the pH-values of their staining solutions directly, but obtained the pH-values of their buffers colorimetrically and assumed that the addition of dye did not alter the reaction appreciably. It is a fact, however, that the addition of dye, particularly of as much dye as ordinarily used, may shift the reaction several tenths of a pH-unit. The glass electrode is the most satisfactory instrument for determining the pH of dye solutions, since hydrogen and quinhydrone electrodes are liable to be poisoned either by the dye itself or by unknown impurities present in it, and since the glass electrode has no tendency to drift. In the present investigation a glass electrode was used to determine the pH-values of the buffered staining solutions.

When a dye stains a protein, the intensity of staining is an index of the amount of dye-protein compound formed. According to the mass law, the amount of dye-protein compound is dependent on the concentrations of dye and protein ions in the reacting system. The concentration of dye ion is a function of the amount of dye present, its dissociation constant, and of pH. Similarly, the concentration of protein ion is a function of the amount of protein present, the isoelectric point of the protein, and the pH of the solution. If, then, dyes are chosen with sufficiently great dissociation constants to be completely ionized, or nearly so, over the pH-range in which they are used, at first glance it would appear possible to determine the isoelectric point of a protein by staining at varying pH-values.

Unfortunately, it is not such a simple matter. The solubility

product or dissociation constant of the dye-protein compound must be taken into consideration. This determines what proportion of the dye-protein compound itself is ionized at any pH-value. An example will show the importance of this factor. Take, for instance, two dye-protein compounds, AP and BP, in which the protein is the same but the dyes are different. At a given pH-value the original concentration of protein ions is the same for both systems. If the dyes selected are completely dissociated at this reaction (they can be selected so that they will be) and if the dye concentrations are the same, the amount of dye-protein compound formed will be the same in both cases. But if the dissociation constant of AP is greater than that of BP, at any pH-value below that at which both are completely dissociated and above that at which neither is dissociated, AP will be more dissociated than BP. In other words, more dye will be ionized from AP than from BP, and consequently the actual concentration of undissociated dye-protein compound will be less for AP than for BP. Hence, the resultant intensity of staining will be less with dye A than with dye B at the same pH. Hence when staining intensity is plotted against pH for the two dyes, different curves will result. Then if one plots curves for two basic dyes and one acid dye, and takes the isoelectric point as the pH at which the acid and basic dye curves cross, two different isoelectric points will be obtained. The more dyes used, the greater the number of "isoelectric points" which will be obtained. The use of different fixatives changes the nature of the proteins, introducing new isoelectric points and new dye-protein dissociation constants. This theoretical reasoning is confirmed by the experimental results obtained in the staining of casein with methylene blue and toluidine blue O (see p. 103), and also by the experimental results given in Table 1. The "isoelectric points" obtained with the different dye combinations range over more than two pH units. This fundamental deficiency of the method makes it impossible to obtain true isoelectric points by its use. No criterion is available to establish which particular combination of dyes gives a true isoelectric point, if any do.

Further confirmation is obtained from Fig. 1 in the paper of Rawlins and Schmidt (1929). Here are plotted titration curves of casein against methylene blue, safranin Y and indulin scarlet for pH-values from 6 to 12. In their experiments pH was established with HCl or NaOH, and not with buffers, and the dye-protein compounds were insoluble even at the high pH-values. The amount of combination was determined colorimetrically. From pH 7 to 12 the amount of dye-protein for the three dyes was approximately the

same. This is to be expected on the basis of the theory. At pH 6, however, the three curves separate. The casein binds 100×10^{-5} equivalents of methylene blue, 150×10^{-5} equivalents of indulin scarlet, and 175×10^{-5} equivalents of safranin Y per gram of protein. Had the curves been carried to a lower pH-value, the results would have been even more striking.

It follows from the mass law that the lower the dye concentration, the less dye-protein compound will be formed at any reaction. Hence, when different concentrations of dye are used, the curves for staining intensity plotted against pH should be different. With basic dyes, as concentration of dye decreases, the curve will be shifted toward a higher pH-value. With acid dyes, the curve will be shifted the other way. As a result, different "isoelectric points" will be obtained with different dye concentrations. A glance at Fig. 2 and Table 2 will show that even with the complex mixture of proteins in protoplasm the above prediction is confirmed.

The buffer system also has its effect on the staining curves. This is because the amount of dye-protein compound is dependent on the concentrations of ionized dye and protein which are free to combine at any reaction. The buffer salts are not inert, but react with both dye and protein. They bind a certain amount of both in the form of un-ionized dye-buffer and buffer-protein compounds. Hence the amount of free dye and protein ions is decreased by the buffer salts. The extent to which this takes place depends on the dissociation constants of the dye-buffer and buffer-protein compounds. The reasoning is similar to that employed for the dye-protein reaction. The smaller the dissociation constant of the dye-buffer compound, the more dye is inactivated by the buffer, and the less will be the quantity of dye-protein compound resulting. Similarly, the smaller the dissociation constant of the buffer-protein compound, the more protein will be inactivated by the buffer, and the less protein will be free to combine with the dye.

The dissociation constants of the dye-buffer and buffer-protein compounds depend in part on those of the buffer salts employed. Hence it would be expected that with different buffers, these dissociation constants would be different and that different amounts of dye and protein would be inactivated. Therefore when staining intensity is plotted against pH, different curves would result with different buffer systems. This is the case in the experiments described in the present paper. "Isoelectric points" almost two pH-units apart were obtained simply by varying the buffer system.

According to the mass law, if the concentration of buffer salts be

increased, more dye and protein should be taken up by the buffer system, and hence less dye-protein compound would be formed. Consequently, with basic dyes, the more concentrated the buffer, the further to the alkaline side the curve of staining intensity will be shifted. For acid dyes, with the more concentrated buffer, the curve will be shifted to the acid side. The experimental results of the present investigation show that this relation holds in the staining of protoplasm.

Temperature, too, plays a part in the reaction, since it changes the dissociation constants of the various compounds present. In short, if the temperature factor is ignored, the intensity of staining depends at a given pH-value upon the interaction of three systems, the dye-protein, dye-buffer, and buffer-protein. If any one factor changes, the intensity of staining also changes.

Any attempt to determine the isoelectric point of a protein by a staining method in the present state of our knowledge would be useless. Even if the dissociation constant of the dye were known, or if it were high enough to produce complete dissociation over the pH-range studied, the isoelectric point would be one of three unknown factors, the other two being the dissociation constants of the dye-protein and the buffer-protein compounds. These would depend in part on the dye and the buffer salts utilized, and therefore when these were varied the results would not be the same.

The above discussion applies to pure proteins. It should be emphasized that protoplasm is not a pure protein, but an extremely complex system of many kinds of proteins, carbohydrates, lipoids and salts. Its protein complex has no true isoelectric point, but rather a more or less broad isoelectric range which is the expression of the isoelectric points of the component proteins. Then, too, the lipoids, carbohydrates and salts are not inert. Reactions between certain dyes and certain of these other constituents would give misleading results. Undoubtedly such reactions occur. It is known that alizarin red S has a strong affinity for calcium. Craig has found (oral communication) that malachite green is precipitated by the phosphate ion. Both calcium and phosphate are always found in protoplasm. Any dye used to determine isoelectric point may, for all we know, have a similar reaction with some specific non-protein ion present in the protoplasm. In such a case it would be expected that different dyes would give different staining results. While the results obtained by Pischinger, Zeiger, the writer and others indicate that cytoplasm and nucleus have different isoelectric ranges and are composed of proteins whose isoelectric points are fairly close to each

other, little more can be said. Since it is at present impossible to find the true isoelectric point of a single protein by the staining method, it would be useless to try to use it to determine actual isoelectric ranges of protoplasmic elements.

Summary

1. The staining reactions at controlled pH-values of various dyes with the nucleus and cytoplasm of *Trichonympha collaris* under different conditions have been investigated. When stain intensity was plotted against pH it was found that:

a) Different curves were obtained for the acid dyes, ponceau 2R and orange G, from those obtained with the basic dyes, toluidine blue O, methylene blue, Nile blue sulfate, and crystal violet.

b) When the concentrations of ponceau 2R and toluidine blue O were varied, the intensity of staining at any pH was a direct function of the concentration of dye.

c) When staining in toluidine blue O and ponceau 2R, stain intensity at a given pH-value was found to be a function of the buffer system in which the staining took place. "Isoelectric points" obtained by superposition of the two dye curves were higher in Sörensen's buffers (sodium citrate and HCl) than in McIlvaine's (citric acid and secondary sodium phosphate), and still higher in Clark and Lubs' (potassium acid phthalate and NaOH).

d) When the concentration of buffer was increased, a lower intensity of staining was obtained than with a more dilute buffer.

2. Various factors modifying staining intensity are discussed from a theoretical point of view. In the staining of a protein in buffered solution, it was shown that staining intensity (the index of the concentration of the dye-protein compound) at a given pH-value is dependent upon the interaction of the dye-protein, buffer-protein and dye-buffer systems, and that as the dye or buffer or their concentrations were varied, the resultant isoelectric points which were obtained also varied. In view of the present lack of knowledge of dyes and dye-protein combinations it would be impossible to determine a true isoelectric point by staining at controlled pH-values without further extensive work on the subject. It follows that the method cannot yet be applied to such a complex system as protoplasm, the aggregate of whose proteins have a more or less broad isoelectric range rather than a true isoelectric point, and which is by no means purely protein in nature.

3. No true isoelectric ranges have hitherto been obtained for nucleus, cytoplasm or other tissue elements by staining at controlled pH-values.

ACKNOWLEDGMENTS

I should like here to thank Dr. Roderick Craig for his helpful advice in the performance of the present research, Dr. Paul L. Kirk for furnishing the pure casein used in this research, and my wife, Helen Saxon Levine, for her assistance in the preparation of this paper.

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A METHOD FOR STAMPING SERIAL NUMBERS ON CELLOIDIN SECTIONS

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One of the best methods of maintaining celloidin sections in serial order is the placement of numbers on the sections. This is a very useful procedure whenever removal of the celloidin is not necessary before mounting as it is relatively simple by comparison with other methods.[1] Furthermore, it allows storing and staining of the sections in bulk without loss of serial identity.

This method as first used by Suzuki[2] and later by Da Fano[3] involves writing serial numbers on the celloidin sections with a fine pointed brush after first cutting and arranging them in sequence on a flat surface. The former used India ink as a writing medium while the latter added ether and acetone to improve the writing quality of the ink.

The procedure to be described facilitates the numbering process by simply stamping the figures on the celloidin with an appropriate ink prior to cutting the sections. This eliminates the task of arranging sections in sequence before marking and in addition the numeration is executed more efficiently with a stamp than a brush. During the past several years more than 10,000 sections of Weigert and Nissl preparations have been handled successfully in this manner.

Success depends largely on employing a stamp ink which is readily absorbed by the celloidin, dries quickly and sufficiently resists fading by alcohols and other reagents. A commercial product bearing the trade name of Triune Opaque Stamp Ink (black)[4] has been found to satisfy these requirements. Rotating rubber number stamps to fit most any need may be procured thru a local stamp dealer. We have,

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[1]Reference is made to general accepted methods such as described in Mallory's Pathological Technique, pp. 61–64. Saunders, 1938.

[2]Suzuki, B. 1909. Eine einfache Schnittserienmethode bei der Celloidineinbettung. Anat. Anz., **34**, 358–61.

[3]Da Fano, C. 1925. On the numbering in series of sections from celloidin blocks. Proc. Physiol. Soc., J. Physiol., **60**, 13.

[4]Triune and Trojan stamp inks are manufactured by the Superior Type Company, 1900 W. Larchmont Avenue, Chicago, Ill. In case this product cannot be obtained locally the company will supply the name of the nearest dealer upon request. The usual price for a two oz. bottle is 55 cents. There may be similar inks made by other companies which would serve equally well.

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however, used a dime store stamp after first removing the year and month bands. This provides numbers 1 to 99 which suffices in most cases since even large series of over 2,000 sections can be conveniently handled in groups of 99 or less.

The ink dries rather rapidly; therefore, it cannot be used on ordinary stamp pads in the same manner as the slower drying inks. The manufacturer recommends a special one (Opaque Stamp Pad) which reduces evaporation and consequent gumminess. A regular (uninked) pad, however, may be substituted if repeated small quantities of ink are applied as needed.

Method: An adequate margin of celloidin must be provided for placement of the numbers while embedding and trimming the block. Good imprints are obtained by keeping the stamp clean and applying the proper amount of ink and pressure. The numbered section is unrolled on the knife just before completing the cut. After completing the cut, the section is floated on water (contained in a wide shallow dish) with the inked surface upward, thus allowing the ink to dry before storing in 70% alcohol. In case only a single section is to be placed on a slide the sections may be stained and mounted without regard to their sequence. If more than one section is to be mounted on a slide, the desired sections must be selected before mounting. The slides may be easily arranged in proper order after placing them against a white background to offer contrast to the numbers on the celloidin. The slides may then be given corresponding numbers and appropriate labels. It has been found desirable to write or stamp on the slides with Trojan[4] ink as it has excellent writing and wearing qualities on glass.

A METHOD FOR INJECTING INSECT TRACHEAE PERMANENTLY

LYLE E. HAGMANN, *Department of Entomology, Cornell University, Ithaca, N. Y.*

ABSTRACT.—By the use of an alcohol insoluble dye (trypan blue), acetic acid, and a detergent ("Santomerse No. 3"), a resulting dye solution is obtained which will completely penetrate the tracheal system of an insect. The dye is injected by the use of a vacuum and by the pressure produced when the air is allowed to re-enter the dye vessel. The dye itself is permanently fixed in the tracheae by means of a fixing solution containing alcohol, acetic acid and barium chloride as its components. The material must be properly preserved after staining. It may be stored indefinitely in 70% alcohol, xylol, cedar oil or clove oil, depending on whether the material is to be used for sectioning or for whole mounts. Injected material may be sectioned in either celloidin or paraffin, or may be cleared and mounted *in toto*.

At present there are only two ways of studying the tracheal system of an insect. One is to cut open the live specimen and examine the air-filled tracheae. The other method is to inject a colored oil into the tracheal system to facilitate observation. In each case the material must be used immediately as both air and oil disperse from the tracheae rapidly. Photographs of uncleared and unmounted material are unsatisfactory, and as permanent staining was not possible prior to this study, drawings were necessarily the only permanent record of research done on the tracheal system.

In the method described below, the injected material may be stored an indefinite length of time after fixation in 70% alcohol, xylol, cedar oil or clove oil, depending on whether the material is to be used for sectioning or for whole mounts. Drawings are not necessary as photographs may easily be made of the slides, which are themselves a fixed record.

To inject insect tracheae permanently the following solutions are required:

Solution A—the dye solution,

Trypan blue[1]	2.0g.
Santomerse No. 3[2]	1.0g.
Glacial acetic acid	10 cc.
Distilled water	90 cc.

[1]Trypan Blue—National Aniline and Chemical Co. Inc. C. I. No. 477. Lot No. 7286.

[2]Santomerse No. 3—A detergent produced by the Monsanto Chemical Co., St. Louis, Mo.

Solution B—the fixing solution,

Formaldehyde (40%)	15 cc.
Glacial acetic acid	10 cc.
Saturated solution of barium chloride in distilled water	75 cc.

Live specimens are chloroformed and placed in a small wire basket. The basket is suspended above the stain by means of a fine wire. This wire is inserted into the aperture of the stopcock, which is then

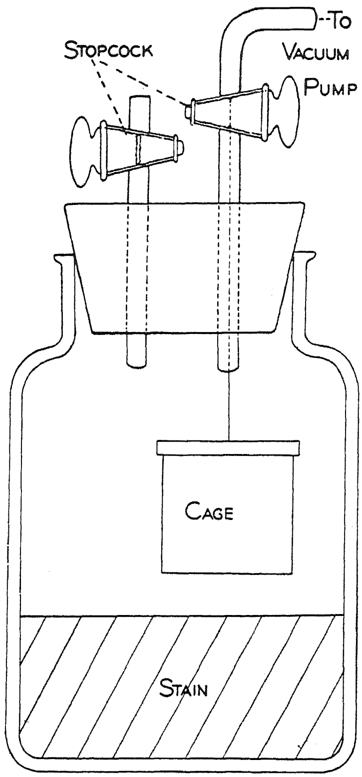

Fig. 1. Diagram of apparatus used to inject stain into insect material.

half closed, pinching the wire and thus holding the basket in place. The air is then exhausted from the vessel containing the stain by means of a vacuum pump. If the water pressure is good a faucet suction pump may be used to create the vacuum. In either case check must be made on the reduction of pressure. The manometer reading should in no case be less than 29 in. of mercury.

After 15 or 20 minutes the basket is dropped into the liquid by opening the stopcock and releasing the wire. The vacuum is maintained for 5 minutes longer and then the air is allowed to re-enter

the vessel slowly thru the other stopcock. (Fig. 1). The specimens are left submerged in the solution until the dye has completely penetrated the tracheae. This requires about 15 minutes.

Upon removal from the dye solution the material is fixed in solution B. Large insects may be fixed more rapidly by injecting the fixing solution directly into the body cavity with a hypodermic

Fig. 2. Photomicrograph of a portion of a celloidin section of the brain of the roach, *Periplaneta americana*, showing the extent of penetration of the stain when injected by the method described. Thickness—20 μ, Magnification—$\times 500$.

needle. This also distends the body sufficiently to prevent distortion caused by muscle contraction.

The specimens should be left in the fixing solution from 3 hours to over night depending on their size. They are then washed briefly in water and upgraded in alcohol to 70% by the usual method for histological material. As the dye is insoluble in alcohol, the stained material may be left in 70% alcohol indefinitely.

Sections may be made either by the celloidin or paraffin method. Safranin makes an excellent counterstain.

Examination of a celloidin section of the brain of the roach, *Periplaneta americana* (Fig. 2), shows the extent of penetration. The tracheal tubes may be traced, under oil immersion, to a point where they measure only 0.2 μ in diameter. This demonstrates that all the tracheae and many of the tracheoles are stained.

The use of this method is limited to those forms which have the holopneustic or hemipneustic type of tracheal system. For this reason many of the larval forms of *Diptera*, *Neroptera* and *Odonata* can not be injected by this method.

NOTES ON TECHNIC

A Simple Method for Mounting Embryological Material. During the course of some experiments conducted by the author for the past 2 years on the demonstration of centers of ossification in various mammalian embryos and fetuses, it became increasingly evident that a suitable method for mounting the specimens for permanent preservation was needed.

The well-known clearing methods utilizing KOH and glycerin were used, together with the respective staining methods for either bone or cartilage preparations. The final process of clearing and preserving was accomplished in pure glycerin (U. S. P.). Since the embryos varied greatly in size (in terms of crown-rump measurement) it was necessary to cut various sizes of glass plates to mount the finished specimens. This proved to be expensive and impractical, especially for material whose crown-rump measurements were less than 30 mm. Resort was finally made to the following procedure:

Sheet celluloid (as used in the automobile industry) was substituted for the glass slides or plates. The convenience of cutting the sheet celluloid to any desirable size and yet have a clarity equal to glass was soon realized. If, for example, an embryo whose crown-rump measurement was 45 mm. was to be mounted in glycerin in a museum jar, it was only necessary to cut the celluloid strip a bit larger and as wide as was needed. By tying the embryo to the celluloid support with the use of thread encircling the body, it was found to hold the specimen in a sturdy but natural position.

In a larger fetus, notches cut in convenient positions on opposite sides of the celluloid mounting strip afforded an adequate support for the specimens.

Buoyancy due to the density of the glycerin is eliminated after a day or so by penetration of the mounting medium into the specimen. —Clarence W. Nichols, Jr., Santa Cruz, California.

Old Gruebler Hematoxylin and Eosin Compared with Current American Stains.—It was the recent good fortune of the writer to be given two bottles of stains marketed by Dr. G. Gruebler & Co. of Leipsig and imported by a Pittsburgh physician sometime before 1908 when they were stored in his attic. As the parchment covers of the brown glass bottles had not been opened the stains appeared to be in perfect condition. One bottle contained 300 grams of "Haematoxylin pur. cryst." and the other an equal amount of "Eosin w. gelblich."

Special interest attaches to these samples because they were purchased during Dr. Gruebler's lifetime and not many years after he left the business. At that time "Dr. Gruebler's Laboratory" (which subsequently became K. Hollborn and Sons) and "Dr. G. Gruebler and Co.", altho existing as separate concerns, were in cooperation[1]. Because of the high repute of the Gruebler stains of those days, a comparison of these with current American stains appeared to be desirable.

The hematoxylin was examined in comparison with a sample of "Hematoxylin C.P." marketed by the Coleman and Bell Co. The Gruebler stain is coarsely granular, the particles varying in size from a powder to masses nearly a centimeter in diameter. In making the traditional .5% solution of hematoxylin the Gruebler stain dissolved more slowly in 95% alcohol, requiring 3 hours for complete solution to 1½ hours for the American stain. The straw color of the alcoholic solutions and of the diluted aqueous solutions was practically identical. On well fixed tissues, using the stains according to Heidenhain's iron hematoxylin method, a definite black staining of the chromatin and of chromosomes was obtained without obscuring coloration of the cytoplasm. The two stains also gave solutions of like color and staining quality when used as Delafield's hematoxylin solution. The results were so nearly identical as to indicate that the two stains are of equivalent staining quality.

The eosin stain is a fine powder similar in appearance to a sample of eosin Y, certification No. NE-4. of the National Aniline and Chemical Company. The Gruebler stain has about the same solubility in water and in 95% alcohol as the "water and alcohol soluble" American stain and the solutions are identical in appearance. Either in an aqueous solution or in an alcoholic solution the stains behaved similarly suggesting near equivalence in quality.

On the basis of these trials it appears that those who use certified American stains today have the equal in reliability of the famous Gruebler[2] stains of the first decade of the century.—T. M. McMillion, Geneva Col., Beaver Falls, Pa.

[1]The writer is indebted to Dr. H. J. Conn for supplying information about the history of the German firms.

[2]Samples of the Gruebler hematoxylin and eosin mentioned have been added to the collection of the Biological Stain Commission. Investigators who wish samples of the stains for comparative studies may obtain them from the writer on condition that the results of trials be reported to the Stain Commission.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

JOHANSEN, DONALD A. **Plant Microtechnique.** 6x9 in. 523 pp. Cloth. 110 illustrations. McGraw-Hill Book Co., Inc. New York, N. Y. 1940. $4.50.

This book, with about 500 pages of text, is perhaps the largest publication that has yet appeared in this country dealing exclusively with the field of botanical microtechnic. It is very attractively put up and illustrated largely from photographs of microscopic material prepared by the author. As he has spent much of his time in recent years preparing and marketing microscopic specimens, the material from which these photographs are prepared is excellent, and his experience with the microtechnical methods involved qualifies him to write on the subject.

The book is divided into two sections: the first of approximately 200 pages on "General Methods", and the second, of about 300 pages, entitled "Special Methods for the Various Phyla". The first of these two sections is a very adequate presentation of the methods employed in plant microtechnic with formulae of the most important solutions used in these methods; two of the sixteen chapters are devoted to stains and staining procedures. The second section of the book contains fifteen chapters, each dealing with special methods needed in the study of one of the large divisions of plants. Altho this is a somewhat unusual method of presenting the subject, it should prove useful to the student in this field.—*H. J. Conn.*

MICROSCOPE AND OTHER APPARATUS

BORRIES, B. v., and RUSKA, E. **Aufbau und Leistung des Siemens-Übermikroskopes.** *Zts. wiss. Mikr.*, **56,** 317. 1939.

This article presents a concise, informative description of the new Siemens' supermicroscope compared with his two earlier models built by the same firm. This instrument is about as compact as a modern X-ray machine and but slightly more difficult to operate. Two photographs of the apparatus with diagrams comparing the conventional and electron microscopes, together with the description of the construction and operation of the latter, are included.—*J. M. Thuringer.*

MASON, W. **Apparatus for cutting frozen sections on the rocking microtome.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums,* **19,** 86–8. 1939.

An apparatus is described for freezing sections which does away with the necessity for a freezing microtome. The object-holder of the rocking microtome is modified to allow of the application of CO_2 by cutting away the jaws into which the wooden blocks are clamped. Upon the resulting flat top and directly over the hole in the center is soldered a metal cap about 3 cm. in diameter by 1.5 cm. deep with holes in the sides which allow CO_2 to escape. The object-holder is attached to the CO_2 cylinder by means of a brass rod 3.5 cm. long, having an external diameter such that it just fits the shaft of the object-holder. A hole 3 mm. in diameter is drilled thru the center of the rod, and the lower part is threaded to fit a brass nut having the same size thread as the CO_2 cylinder. If the gas is applied in a steady gentle stream, the interior of the cap fills with CO_2 snow and the tissue will remain frozen long enough for sectioning.—*Jean E. Conn.*

REESE, J. D. **A useful apparatus for staining slides.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 83–5. 1939.

An apparatus has been designed for staining slides which enables one to adjust the rack to a horizontal position and permits the dye to be poured back into the bottle without waste. A ring stand is used which has an upright bent at right angles about 2 in. from the end. A clamp holder is attached to the bent portion. The slide holder is made of stout brass wire, rectangular in shape, with a short metal rod attached to one end. By placing the rod in the free arm of the clamp holder, the rack may be easily adjusted to the horizontal. Small knobs of solder placed at intervals of about an inch along the rack keep the slides apart. A piece of monel metal with small metal "stops" opposite the spaces for the slides is soldered to one side of the rack in a slightly inclined position. When the rack is tilted, this piece of metal acts as a drain allowing the dye to flow down the incline into a bottle.—*Jean E. Conn.*

PHOTOMICROGRAPHY

KROGH-CHRISTOFFERSEN, A. **Das Mikrophotographieren ohne photographische Geräte.** *Zts. wiss. Mikr.*, **56**, 301. 1939.

Photomicrography, without the use of cameras, by projection of the microscopic image directly upon suitable projection paper is described. A contact print from the paper negative obtained produces the finished picture. Any one interested in this method is referred to articles on the well-known paper negative processes.—*J. M. Thuringer.*

SMITH, HILTON A. **A technique for making photomicrographic prints in color.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 45–51. 1939.

The author describes a method of adapting Eastman's "Wash-off Relief Process" to photomicrography. Since most sections are stained only in two colors, they can be reproduced by using only two negatives instead of three as is usually required. This method is described for tissues stained with hematoxylin and eosin, altho it may be adapted for other stains by using different filters. The A negative is exposed with the red light of two Wratten filters in combination, B (No. 58) plus E (No. 22). The corresponding positive is printed in the complementary color, a shade of blue, and reproduces the hematoxylin-stained nuclei. The red eosin is reproduced by the B negative which is exposed thru green filters. The writer found B (No. 58) plus H (No. 45) to be satisfactory; the complementary color, used in printing, is of eosin shade. The type of film preferred by the author is Process Panchromatic for the A negative and Panatomic for the B negative.

Chromatic aberration may be prevented by making a slight change in length of camera bellows between the two exposures. With a Bausch and Lomb type H camera, a 16 mm. aprochromatic objective and an 8× compensating eyepiece, the bellows will need to be about 1.5 mm. shorter for the B picture than for the A. The exact amount, however, must be determined by experiment.

In selecting dyes, Eastman's "A" dye (blue) was found satisfactory, but not the "B" dye. To replace the latter, the writer uses a mixture of orange G (C. I. No. 27), 2 vol., Bordeaux red (C. I. No. 88), 1 vol., and amaranth (C. I. No. 184), 1 vol. The total mixture is used in a 0.3% aq. solution.

For further details the author refers to publications of the Eastman Kodak Co. —*Jean E. Conn.*

DYES AND THEIR BIOLOGICAL USES

HÖBER, R. and BRISCOE-WOOLLEY, P. M. **Conditions determining the selective secretion of dyestuffs by the isolated frog kidney.** *J. Cellular & Comp. Physiol.*, **15**, 35. 1940.

The influence of the molecular configuration of a number of sulfonic acid dyes of the mono-azo, dis-azo and triphenylmethane groups on their secretion by the proximal tubules of the isolated Ringer-perfused frog kidney has been studied.

The sulfonate derivatives of mono-azo and dis-azo dyes are secreted by the kidney tubules and are found in the secretion in a concentration higher than that injected into the renal portal vein, provided that the sulfonate groups are arranged in a special manner. The triphenylmethane dyes do not undergo any secretory transport across the kidney tubules. Lipoid solubility of the dyes favored their secretion but was not indispensable.

From the data it appears that for active secretion of the dyes to occur, a bilateral elongated molecule, with the sulfonate groups on one half of the molecule, is necessary, whereas if sulfonate groups are located at the two ends of the molecule, the secretion of such dyestuffs is prevented. It is suggested that the first stage in the process of secretion is the anchoring of the dye on the cell surface by the organophilic non-polar portion of the molecule, the hydrophilic polar part (containing the sulfonate groups) attaching at the aq. surface.—*L. Farber.*

KROLL, H., STRAUSS, S. F., and NECHELES, H. **Concentration and detection of a dye in abscesses.** *Proc. Soc. Exp. Biol. & Med.*, **43**, 228–34. 1940.

To 100 mg. of an acid dis-azo dye, T 1824 (Evan's blue), in 15 cc. of water, aq. bromine was added, drop by drop, until the theoretical amount (2, 3 or 4 atoms) had been taken up. This was injected intravenously into dogs having abscesses, produced within 24 hr. after the injection of bacteria and foreign material. The abscess tissue was analyzed 20 hr. later. Best results were secured with monobrominated dye. Fixation of the dye by abscess tissue was regarded as due to absorption on the cell membrane and retention by solution of dye in the lipoid material of the cell wall.—*M. S. Marshall.*

LISON, L., and FAUTREZ, J. **L'étude physicochimique des colorants dans ses applications biologiques.—Etude critique.** *Protoplasma*, **33**, 116–51. 1939.

This is a critical analysis of the way in which many biologists have used dyes with little or no understanding of the varied properties of the dyes and have drawn conclusions which tend to be erroneous. An example given is the use of dyes in testing the lipoid nature of cell membranes by comparing the penetrability of the dyes with their solubility in fatty materials, without appreciating the variability of dye solubility in different fats and oils. There are also the investigations on the ultra filter theory by comparing intracellular penetration with the diffusibility of the dyes in solution without considering the nature of the diffusion medium. The authors stress the importance of understanding the physicochemical properties of the dyes, especially in biological media.

The length of the article limits a description to an enumeration of the chapters as follows: (1) Nomenclature and classification; (2) Chemical reactions and transformations, e.g., reducibility, formation of basic carbinols (pseudobases) and metachromasia; (3) Diffusibility and dispersibility in water, gels and dialysis; (4) Liposolubility, varying according to the lipoid material used and the possibility of hydrophily of some lipoids; (5) Electric charges; (6) Flocculability; (7) Electrocapillarity and surface activity (tensio-activité); (8) Physicochemical modifications in colloidal media, especially in serous media. A list of over 100 references is appended.—*Robert Chambers.*

MIRIMANOFF, A. **Remarques sur la secretion des tentacules de Drosera. Notes histochimiques.** *Protoplasma*, **33**, 211–14. 1939.

The author tested the reaction of the secretion to various agents by several methods, the best of which he found to be the following. Filter paper was moistened with a given reagent, then dried and a leaf of Drosera placed on the filter paper so that only the tips of the tentacles were in contact with the filter paper. Any reaction which took place at the points of contact was considered to be the result of the secretion at the tips of the tentacles. The reagents used were silver nitrate, parachlor-indophenol, o-nitroso-nitro benzol, ferric chloride, sodium nitro-prussate, Fehling's solution and potassium ferricyanide with ferric chloride. All showed the presence of a reducing substance. The results obtained convinced the author that the secretion contains ascorbic acid.—*Robert Chambers.*

ANIMAL MICROTECHNIC

COOKE, JEAN V., and BLATTNER, RUSSELL J. **Vital staining of virus lesions on chorio-allantoic membranes by trypan blue.** *Proc. Soc. Exp. Biol. & Med.*, **43**, 255–6. 1940.

One cc. of trypan blue, 0.5% aq., is placed on the membrane of the infected embryo; the egg is gently rotated and incubated 10–30 min. The membrane is removed, washed in saline and fixed flat in 10% formalin for a few minutes. The membrane is then drained, flattened on a 2x2 in. slide and mounted in glycerin gelatin, (50% glycerin, 5% gelatin, 1% phenol). Mounting is done at 70° C. The edges are sealed with balsam or asphalt.—*M. S. Marshall.*

INGLEBY, HELEN, and HOLLY, CLAIRE. **A method for the preparation of serial slices of the breast.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 93–6. 1939.

The following method is described for making serial sections from whole breasts: Fix breast by suspending it in a gauze sling in a jar of 4% formaldehyde; leave jar at least 24 hr. in the refrigerator room; remove breast from solution, wrap in a wet cloth and freeze. (The commerical ice cream freezer is handy for rapid freezing. The slicer put out by the American Slicing Machine Co. is the most satisfactory for making the serial slices.) After slicing, pile sections in order. (Difficult sections may be received in a bowl of water, floated onto numbered pieces of paper and stacked.) Fix sections a few hours; rinse in dist. water; stain 24 hr. in a single layer in a filtered solution of 5 cc. Harris' hematoxylin in 100 cc. dist. water; turn once to insure even staining; differentiate in acid alcohol; wash until blue; 70% alcohol, 1–2 hr.; 95% alcohol, 6–8 hr. (2 changes); abs. alcohol, 2–4 hr. (2 changes); benzine, 2–4 hr. (2 changes); store sections in mineral oil until required for use. Then transfer to oil of wintergreen (2 vol.) and benzol benzoate (1 vol.) for about 1 hr. before examining under the dissecting microscope. Masson's iron hematoxylin is an alternative stain recommended if photography is contemplated. After study sections must be returned to mineral oil.

The advantages of this method are: Every part of the tissue may be examined grossly; a three dimensional view is obtained; a whole breast can be ready for examination in 5 days; paraffin sections can be made at any time from any part if higher magnification is desired.—*Jean E. Conn.*

JALOWY, BOLESLAW, and CHRZANOWSKI, BRONISLAW. **Einige Bermerkungen über den Vorversilberungsprozess.** *Zts. wiss. Mikr.*, **56**, 334. 1939.

The authors review the theories advanced concerning the action of silver salts in impregnation processes (primary and secondary impregnations). Their work deals with the action during primary impregnation of tissues (preargentation as distinguished from postargentation when the silver salts are applied after preliminary treatment with suitable fixative or mordant). They conclude, on the basis of their investigation, that during preargentation the chlorine ions play the exclusive role in forming light sensitive AgCl.

They demonstrate that chlorine ions could be rapidly removed from the mesentery and other tissues by rinsing in large quantities of dist. water or by previous fixation with 10% or 20% formalin.

The AgCl formed during preargentation is converted into metallic silver under the action of light. This process does not begin spontaneously and when inaugurated thru light does not progress further. Pure silver albuminates do not darken with the action of light; their role is limited to the formation of a stroma (substrate) in which the granules of metallic silver are deposited. Silver phosphate, ortho-, meta-, and pyro-phosphate are likewise insensitive to light.—*J. M. Thuringer.*

KRAMER, FRANK M. **Macroscopic staining of anatomic and pathologic specimens.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 72–8. 1939.

Methods are described for the macroscopic staining of fat, iron, calcium, amyloid, and the brain. The following method is recommended for staining fat: Fix in formalin; cut a fresh surface and immerse in a sat. solution of Sudan III

or "scharlach R" (i.e. Sudan IV) in 70% alcohol until the fat has assumed a brilliant red color; avoid overstaining; decolorize in 95% alcohol for 12 hr. or more; wash thoroly in running water; mount in weak formalin. Fat-products of degeneration are also stained, but normal fat is stained more intensely.

For iron, the Berlin blue method is recommended as follows: Place specimen in a freshly prepared solution of equal parts 2% $K_4Fe(CN)_6$ and 1% HCl, until the desired reaction takes place; wash; preserve or mount in 70% alcohol.

The following silver nitrate method is recommended for staining calcium: Wash formalin-fixed specimen at least 24 hr. in running tap water; process thru several changes of dist. water for about 24 hr.; stain in the dark for 6–15 hr. in 1% $AgNO_3$ in dist. water; rinse in dist. water without exposing to light; place for a full day in 5% hypo solution; wash thoroly; mount in 50% alcohol or Kaiserling's preservative. Calcium may be stained a vivid pink color by alizarin dyes as follows: Stain 12 hr. in a 1:10,000 solution of alizarin red S, made basic by adding a small amount of KOH; differentiate for several days in equal parts of alcohol and glycerin, exposing jar to sunlight; mount in an alkaline preservative, e.g. Kaiserling's with a small amount of KOH added (1:1000).

Immersion in Lugol's iodine solution stains amyloid an intense brown, made sharper by adding weak H_2SO_4. To prevent fading, equal parts of 2% Lugol's and 1% H_2SO_4 are used as preservative.

The best method for staining the brain is as follows: To prevent tinting of the white matter, first process the specimen for a few min. in a phenol solution (phenol crystals, 80 g.; HCl, 3 cc.; $CuSO_4$, 10 g.; dist. water, 2000 cc.); immerse in cold tap water; stain in 0.5% nigrosin solution; rinse and examine occasionally to determine progress of staining; when the correct intensity is reached, remove "excess" dye with weak alcohol; rinse in running water; mount in weak formalin. This is the only method in which the intensity of staining can be favorably controlled.—*Jean E. Conn.*

LENDRUM, A. C., and McFARLANE, D. **A controllable modification of Mallory's trichromic staining method.** *J. Path. & Bact.*, **50**, 381–4. 1940.

By applying the stains of the Mallory technic one at a time the effects desired may be attained with greater assurance. For the nuclear stain the following procedure is used: Allow 2.5 g. iron alum to dissolve over night at room temp. in 50 cc. dist. water. Add to this 0.25 g. celestin blue (C. I. No. 900) and boil the mixture 3 min. Filter when cool, and add 7 cc. glycerol. Stain in this solution 10–20 min. Rinse in water. Add filtered Mayer's hemalum; stain 5–10 min. Rinse in 95% ethanol. Differentiate in acid alcohol (1% HCl in 95% ethanol) until the red color ceases to come off.

After this step, the cytoplasm is stained as follows: Treat with 0.2% orange G in 80% ethanol saturated with picric acid, 2 min. to 16 hr. Rinse in water 30 sec. to 2 min. From a filter add fuchsin-ponceau solution (prepared by mixing 1% acid fuchsin in 1% acetic acid with 1% ponceau 2R, C. I. 79, in 1% acetic, and adding 2.5 cc. of 10% Na_2SO_4 to each 100 cc. of dye solution to inhibit mold growth). Stain for 15 sec. to several min. Rinse in 1% acetic acid and examine. Decolorize connective tissue in 1% phosphomolybdic acid; do not decolorize completely. Tissue fixed without chromate is sufficiently decolorized in 1–2 min. Stain in 2% soluble blue (C. I. 706) in 1% acetic acid, 2–10 min.; or in fast green F.C.F. in 1% acetic, 2–10 min. Rinse in 1% acetic acid and examine; if satisfactory, dehydrate rapidly and mount.

By this technic the nuclei stain dark red and the blood is colored distinctly different from the fibrin. This nuclear stain is more resistant to the action of picric acid than iron alum hematoxylin. The celestin blue solution should be replaced every 6 months; the other stain solutions last longer. The nuclear stain may appear to be entirely removed in the acid alcohol, but a mordanting effect is achieved in the nucleus that is not affected by picric acid and which fixes the acid fuchsin. Excess fuchsin staining may be remedied by immersion in the picric-orange-G solution. To emphasize color differences for photography, sections may be mordanted before staining in Hollande's solution without acetic acid. Poorly stained or faded sections can be re-stained by immersion in picric orange G or in a saturated solution of picric acid in 80–100% alcohol for 30–60 min., then rinsed and restained. Biebrich scarlet can replace the fuchsin-ponceau mixture. "Revector" dyes were used by the writer.—*S. H. Hutner.*

LILLIE, R. D. **The effect of hydrogen-ion concentration of formaldehyde used in storage for varying periods on staining of tissue.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 89–92. 1939.

The author reports experiments which show that formaldehyde solutions buffered to pH 7.5 preserve staining reactions of tissues for Romanowsky staining in particular and for nuclear staining in general much better than does formaldehyde not so buffered. The need for further work on this point is indicated.—*Jean E. Conn.*

MICHAEL, E. G. **Rapid method of staining frozen sections of tissues requiring immediate diagnosis.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 47–9. 1940.

The writer recommends the following rapid technic: Put tissue in 20 cc. of 10% formalin, boil 1 min. Cut sections and put in dist. water in a Petri dish. Put a section on a slide in dist. water in another Petri dish. Blot, cover with celloidin in alcohol-ether, and blot again. Put in dist. water, 20 sec. Remove. Cover with filtered Delafield's or Ehrlich's hematoxylin, 20 sec. Rinse in dist. water. Cover with 1% eosin Y in 95% alcohol, 15 sec. Add 3 drops of Mallincrodt's new beechwood creosote without removing the eosin. As soon as it penetrates, add 1 drop of Canada balsam in xylol and apply a cover slip. Wipe off excess mixture of eosin, creosote and balsam with a piece of gauze moistened with xylol.—*G. H. Chapman.*

ÖKLAND, FRIDTHOF. **Untersuchungen über Osteoblasten in Schliffen und Ausstrichen.** *Zts. wiss. Mikr.*, **56**, 345. 1939.

This work is based on the Rupprecht and Krompecher methods of preparing ground bone sections with combined fixation and staining. The author suggests the following modifications. Thin pieces of cranial vault of mouse, rat, or guinea pig (5 mm. sq.) are fixed for 24 hr. each in 60%, 95%, and abs. alcohol, and xylol. They are then ground on small carborundum hones saturated and kept wet with xylol. The thin sections are rinsed in clean xylol and brought into abs. alcohol, 5 min.; methyl-green-pyronin (Pappenheim, Grübler), 24 hr.; abs. alcohol, 10 min.; xylol, 1 hr.; neutral balsam. Results may be varied by previous *in toto* staining with Nile blue sulphate (sat. solutions in 60%, 95%, and abs. alcohol), then proceeding as above including the methyl-green-pyronin stain after grinding sections.

Bone marrow smears for control preparations may be stained with May-Gruenwald (similar to Wright's) or in the following way: a) methyl-green-pyronin, 3–5 min.; b) rinse in dist. water; c) abs. alcohol, xylol, and neutral balsam.

Results: cytoplasm stains red; characteristic vacuole, colorless.—*J. M. Thuringer.*

PARMENTER, C. L. **Chromosome numbers in Rana fusca parthenogenetically developed from eggs with known polar body and cleavage histories.** *J. Morph. and Physiol.*, **66**, 241–60. 1940.

Modifications of Bouin's solution (B-3 and B-15 without urea) are used in fixation. Since alcohols shrink the jelly into an unremovable, thin, tough coat, the eggs should be washed and preserved in 4% formalin, with frequent additions of Li_2CO_3 during washing. The following procedure is recommended: Before embedding, place eggs in 35% alcohol and dissect jelly from the eggs with very fine insect needles. Dehydrate and clear eggs in 30-min. successive baths of ⅓ dioxan, ⅔ dioxan, and 2 changes of pure dioxan. Infiltrate successively in equal parts of dioxan and soft paraffin at 47° C., soft paraffin at 47° C., and hard paraffin or tissuemat at 56° C. for about 15 min. each. Section eggs at 13 μ, with the knife passing thru both poles simultaneously. Of several stains used, Heidenhain's hematoxylin without counterstain, and safranin followed by light green or fast green give the most satisfactory results.—*Elbert C. Cole.*

PERRY, I. H., and LOCHHEAD, M. S. **Histological technique for the pituitary gland of the mouse.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 101–3. 1939.

A reliable differential stain for the pituitary of the mouse is described as follows: Remove the gland as rapidly as possible from the anesthetized animal by reflecting back the skin over the head and cutting the skull from its base. Cut out a wedge of bone around the pituitary and put into the fixing solution without handling. After hardening 15–30 min., dissect from the bone with needles under a binocular microscope without removing from the fixative. Transfer the pituitary with a pipette.

Fixation and embedding: Fix 4 hr. in Zenker-formaldehyde (95 cc. Zenker plus 5 cc. neutral 39% formaldehyde. Add the formaldehyde to the Zenker immediately before use). Wash 4 hr. in dist. water (many changes); dehydrate in 50% and 60% alcohol for ½ hr. each; leave over night in 70% alcohol; 70% iodized alcohol, 1 or 2 hr.; 80%, 90% and 100% alcohol, 2 hr. each; ether-alcohol, 2 or 3 hr. at 36° C.; one day each in 10%, 25% and 50% nitrocellulose, or 2%, 4%, 6%, 8% and 16% celloidin; harden, and cut at 4 μ.

The slides are stained using the technic described by Koneff (Stain Techn., **13,** 49–52, 1938) with the following modifications: Before staining, leave 12 hr. in 3% potassium bichromate, and rinse in dist. water. In step 1 treat with anilin alcohol 18 hr. instead of 45 min. In step 3 leave in azocarmine solution 4 hr. at 56° C. and 14 hr. at room temp.

The results are: Basophiles light blue with bluish-red nuclei; two types of acidophiles shown, one stained orange, the other deep red; nuclei of the acidophiles pink, bluish red or dark red; chromophobes gray with nuclei usually red; mitochondria in the basophiles always stained red.—*Jean E. Conn.*

SCHALM, O. W., and HARING, C. M. **A technique for reducing soft-tissue organs to thin serial slices, with special reference to its use on bovine mammary glands.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19,** 97–100. 1939.

A technic is described by which the entire bovine udder may be reduced to serial slices. The procedure is as follows: Remove the entire mammary gland at slaughter; milk out as much secretion as possible; inject the duct and vascular systems with 8% formaldehyde, using a 5-liter aspirator bottle fitted with a rubber bulb for raising the pressure in the bottle. Inject the vascular system first thru the mammary artery of each mammary half; ligate the stumps of the large vessels as the formaldehyde escapes; continue injection until all the vessel stumps are so closed. Inject the duct system of each quarter thru the teat canal until the quarter is distended and tense. A round wooden applicator may be inserted into the teat canal to avoid distortion during hardening. Suspend the entire udder in 4% formaldehyde until thoroly hardened (2–6 weeks); separate the mammary halves along the median line; trisect the halves by cutting thru the center of each quarter from the tip of the teat to the base of the gland; slice each piece in an electrically-driven meat-slicer, making the slices from 3–5 mm. in thickness; attach paraffin-coated tags to each slice for identification; wrap together in cheesecloth; store in 4% formaldehyde. The procedure may be suspended at any stage as long as the tissues are kept submerged in paraffin.

The thin slices may be dehydrated in a graded series of either dioxan or tertiary butyl alcohol and infiltrated with paraffin. The slices are cooled between two glass plates, excess paraffin removed by gentle heat, and a protective coat of white shellac applied. Slices so prepared are best observed by transillumination.—*Jean E. Conn.*

VRAA-JENSEN, G. **Eine Method zur Doppelfärbung von Übersichtspräparaten des Zentralnervensystems.** *Zts. wiss. Mikr.*, **56,** 356–8. 1939.

This method is suited for "survey sections" of the central nervous system with excellent contrast between myelinated fibers and nerve cells. Sections are cut in paraffin (15 μ) and celloidin (20–25 μ).

Technic: 1) Deparaffinize. 2) Dist. water, 2 changes. 3) Stain in iron hematoxylin at 50–55° C. (paraffin sections, 10–15 min., celloidin sections, 20–30

min.). Prepare by mixing equal parts of 4% aq. $FeNH_4(SO_4)_2$ and 1% hematoxylin solution (10 ml. ripened solution hematoxylin to 90 ml. dist. water) immediately before use. 4) Tap water, 2 changes. 5) Differentiate in 4% aq. $FeNH_4(SO_4)_2$ (paraffin sections until the blue color is no longer visible thru the under surface of the slide; celloidin sections until the celloidin is colorless). 6) Tap water, 3 changes. 7) Differentiate, controlling with microscope, until cells just lose their stain in the following solution: 10 g. sodium borate, 12.5 g. $K_3Fe(CN)_6$ to one liter of dist. water. 8) Dist. water, 2 changes. 9) Rinse 5–10 min. in Vlesschouwer's pH 11 buffer solution (97.3 ml. of 0.53% aq. Na_2CO_3 and 2.7 ml. of 1.91% aq. $NaBO_3$). 10) Stain paraffin sections, 4 hr., or celloidin sections, 2 hr. in the following solution: Dissolve 0.1 g. Kernechrot (Hollborn), in 100 ml. 5% $Al_2(SO_4)_3$ by heating slowly, stirring constantly, and bringing to boil for 5 min.; allow to cool to room temp. and filter; add sufficient water thru the filter to bring the filtrate to 100 ml.; for every 5 vol. of the stain add 1 vol. $N/1$ NaOH. 11) Dist. water, 2 changes. 12) Dehydrate thru xylol and mount in balsam.

Results: Myelin sheaths, dark blue; nerve cells, connective tissue, non-myelinated fibers, and nuclei of neuroglia stain red; nerve cells stain most brilliantly while the intensity of the other structures gradually diminishes in order given. In the cerebrum the best results are obtained with celloidin.—*J. M. Thuringer.*

PLANT MICROTECHNIC

TANAKA, N. **Chromosome studies in Cyperaceae. VI. Pollen development and additional evidence for the compound chromosome in Scirpus lacustris L.** *Cytologia*, 10, 348–362. 1940.

In connection with a cytological study of pollen development, the following recommendation is made:

After aceto-carmine stains, seal the cover-glass in place with melted "valap", a mixture of 2 parts vaseline, 2 parts lanolin, and 1 part paraffin, applied with a glass rod. If necessary, remove the valap with chloroform.—*Virgene Kavanagh.*

MICROÖRGANISMS

BRUNER, D. W., and EDWARDS, P. R. **Application of the endospore stain to blood smears from opsonophagocytic tests.** *J. Lab. & Clin. Med.*, 25, 543–4. 1940.

This stain permits an accurate count of phagocytosed bacteria. A smear is prepared from the test material as follows: Spread the contents of a 2 mm. loop over 4 sq. mm. of a slide; air dry; fix by flaming 3 times; apply 5% aq. malachite green, 5 min.; wash 10–20 sec. in tap water; apply 0.5% aq. safranin, 10 sec.; wash quickly with tap water; dry and examine. The method is not applicable to all species of bacteria, for some do not retain the green in the presence of the safranin. Staphylococci are stained red. Streptococci are stained red for the most part but a few cells stain green. Some Gram-negative bacilli are stained red and others green. This technic gives excellent contrast between bacteria and white blood cells.—*John T. Myers.*

CANSEY, O. R. **Description of three species of frog microfilariae with notes on staining methods.** *Amer. J. Hyg.*, 30, 117–21. 1939.

In the course of the writer's investigations of infected frogs, four stains for blood smears were employed: (1) Hemalum. Dried smears were dehemoglobinized in saline, placed in warm 70% alcohol for 15 min., passed thru tap water, then thru slightly alkalinized water and placed in the stain for 3–5 hr. They were destained in 70% alcohol containing 1% HCl until differentiated. After washing in tap water they were passed thru 70, 80, 95 and 100% alcohol, cleared 5 min. in xylol and mounted in balsam. (2) Methyl-green-pyronin: 0.2 g. methyl green and 0.075 g. pyronin in 100 cc. of isotonic NaCl solution. After being dehemoglobinized in saline, smears were placed in the stain for 12–36 hr., washed and mounted. (3) Azure-II-eosin. A stock solution of azure II was prepared

by adding 1 cc. each of 1% aq. azure II and 1% aq. Na_2CO_3 to 500 cc. of saline; this was diluted in 24 vol. of saline for staining. Dehemoglobinized smears were stained in the dilute azure for 3–5 hr., removed, and without washing, several drops of 0.025% eosin in saline added. A cover-slip was sealed in place with vaseline. (4) Vital azure II. Fresh blood was placed on a slide on which several drops of the stock azure II of No. 3 had been dried. After mixing, a cover-slip was put in place, and examination was made while fresh.—*John T. Myers.*

FITE, G. L. **The fuchsin-formaldehyde method of staining acid-fast bacilli in paraffin sections.** *J. Lab. & Clin. Med.*, 25, 743–4. 1940.

The most reliable means of staining acid-fast bacilli in sections is by the following method, washing in tap water between each step: Place in a solution, containing new fuchsin 0.5 g., phenol crystals 5.0 g., alcohol (methyl or ethyl) 10 cc. and water to make 100 cc., for 12–24 hr. at 60° C. or 24–48 hr. at room temp.; transfer to freshly dist. aq. 5–30% formaldehyde, 5 min.; place in alcohol containing 2% of HCl, 10 min.; 1% aq. $KMnO_4$ until brown, usually 2–5 min.; 2% aq. oxalic acid, 1 min.; stain 2 min. in Harris' hematoxylin; then in Van Giesen's stain (acid fuchsin 0.1 g., picric acid 0.5 g., dist. water to make 100 cc.). Without washing, dehydrate in alcohol, clear in xylol and mount in balsam. Nuclei stain brown, connective tissue fibers red, muscle fibers yellow, and acid fast bacilli dark ultramarine blue. Lepra bacilli impossible to demonstrate by other methods were readily stained.—*John T. Myers.*

HAKANSSON, E. G. **A method of destroying the blastocysts (Blastocystis hominis) in fecal wet smears in order to facilitate the examination of Endamoeba histolytica.** *J. Lab. & Clin. Med.*, 25, 546–7. 1940.

Blastocysts can be ruled out by making the fecal preparations in water instead of isotonic saline. Blastocysts and protozoon trophozoites will disintegrate but cysts will remain for several hours.—*John T. Myers.*

HORNUS, G. J. P. **Psittacose pulmonaire expérimentale de la souris blanche.** *Ann. Inst. Pasteur*, 64, 97–116. 1940.

The following procedure is recommended for staining elementary bodies concentrated from emulsified infected lung: Smear suspension on a slide; dry; fix with methyl alcohol; when nearly dry, rinse thoroly in tap water; stain in 1% toluidine blue. Rinse; differentiate in 1% eosin-orange (neither formula nor source of dye stated); rinse, dry and examine. Five min. in toluidine blue suffice for recognition of the elementary bodies; longer staining gives more intense colors. The bodies are violet blue.

A modified Dominici technic is described for staining elementary bodies in sections. Fix in Bouin; cut paraffin sections and bring down to water; mordant ½ hr. in Lugol's solution; decolorize with $Na_2S_2O_3$; rinse in water. Stain ¼ hr. in a mixture of 1% erythrosin, and 1% orange G. Wash in running water; stain 5 min. in 1% toluidine blue. Differentiate in 0.2% acetic acid until the sections are rose-colored. Dehydrate and mount. Protoplasm of the cells is rose, with nucleus violet and elementary bodies clear blue.

An alternative Giemsa method is as follows: Prepare sections after Bouin fixation; rinse in buffer (2% Na_2HPO_4, 1% KH_2PO_4). Stain in Giemsa solution freshly prepared by adding 25 drops of dye to 25 ml. neutral water. Stain 12 hr., then an additional 36 hr. in fresh stain solution. Wash in running water; differentiate in a mixture of 1% orange G and 5% tannic acid until the section has an orange tint. Change the latter solution once or twice as it becomes saturated with the Giemsa stain. The results are cytoplasm yellow, nuclei brown, and elementary bodies intense violet. A color plate illustrates these effects.—*S. H. Hutner.*

JOHNS, C. K., and HOWSON, R. K. **Potentiometric studies with resazurin and methylene blue in milk.** *J. Dairy Science*, 23, 295–302. 1940.

Coefficients of correlation of the reduction test times and the Breed counts of individual cells on 369 samples of market milk were $r = -0.711 \pm 0.017$ for the

resazurin test, using a definite pink color as the end-point, and r = −0.651 ± 0.020 for the methylene blue test. The tubes were inverted every two hours in the reduction tests. The ratio of the resazurin reduction times to the methylene blue reduction times was approximately 3:4. The shapes of the time-potential curves obtained with the two dyes indicate that the shorter reduction time with resazurin is due to the more rapid initial drop in potential which occurs. Shortly below the point where the change to pink takes place, the curve flattens somewhat before again declining to reach the same final potential level as the milk to which methylene blue was added. In the range immediately following reduction to the pink, resazurin seems to have a greater poising action than does methylene blue at the same level of potential. More rapid reduction as a result of inversion of the tubes at hourly intervals is due to more active growth of bacteria. The resazurin test apparently is more satisfactory than the methylene blue test for grading samples containing considerable numbers of slow-reducing thermoduric organisms.—*F. E. Nelson.*

MATUSZEWSKI, T., and SUPINSKA, J. **Studies on the methylene blue reduction test. II. Comparison between the old and the modified methods.** *J. Dairy Research*, **11**, 43–50. 1940.

Methylene blue reduction tests were made on each of 185 milk samples, using both the old method and the new method of Wilson, in which the tubes are inverted every half hour until reduction occurs. The new technic gave a shorter reduction time than the old, and smaller variations in the numbers of bacteria (determined by microscopic examination of stained preparations) corresponding to a given reduction time. The numbers of organisms actually present in the milks at reduction time approximated very closely the numbers calculated by means of equations whose derivation is presented. More bacteria were present at the time of reduction in the inverted tubes. Abnormally short reduction times, possibly due to aeration before the test began, and unusually long reduction times, apparently due to slowly active bacteria, were encountered at times. The authors believe the proposed modification constitutes an advance in the reduction test technic.—*F. E. Nelson.*

PARSONS, R. J. **The staining of Negri bodies in formaldehyde and alcohol fixed tissues.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 104–8. 1939.

The following procedure is described as a simple and reliable stain for Negri bodies in tissue fixed in formaldehyde or alcohol: Fix in 4% formaldehyde 18 hr. to 7 weeks or in 96% alcohol 18 hr. to 3 weeks. (It is preferable to dissect out the hippocampal gyrus before fixing and to prepare several cross-sectional blocks. The cerebellum should always be fixed.) Float paraffin sections cut at 4–5 μ directly onto clean cover slips or slides; dry over night in a warm oven or warm over a flame until the paraffin just begins to melt; remove paraffin from sections and bring to 96% alcohol. Stain 2 min. in acidulated ethyl eosin prepared as follows: Add 1.25 cc. of a solution of 1.0 g. ethyl eosin (National Aniline & Chemical Co., certified) dissolved in 100 cc. ethyl alcohol 96% and filtered before use to 22 cc. of a solution of 0.6 cc. acetic acid (Reagent Special, 99.5% acetic acid) in 99.4 cc. dist. water. Wash off excess stain in 96% ethyl alcohol, leaving sections a medium pink color. Stain 2 min. in borax-methylene-blue (methylene blue, 1.0 g.; borax, 1.0 g.; dist. water, 100 cc.). Rinse briefly in dist. water. Differentiate 2–5 min. in 0.25% acetic acid (99.5% acetic acid, 1.0 cc.; dist. water, 400.0 cc.), making sections a pale bluish-pink. Rinse in 96% alcohol; dehydrate as rapidly as possible in 2–3 changes of abs. alcohol; xylol (2 changes); mount in balsam.

Results: Negri bodies bright orange-red with a vacuolated inner structure or a small blue central dot; Lyssa bodies bright orange-red, but uniformly stained; nucleoli dark blue; red blood cells an intense copper red; nuclei blue; Nissl substance bright blue; cytoplasm of nerve cells pale pink; neuroglia pale pink. The differential coloration of the Negri bodies is so striking that they can be found under low (×100) magnification, using higher magnification only for positive confirmation. This stain has been employed successfully on material from dogs and man.—*Jean E. Conn.*

STEINER, GABRIEL. **A simple method of staining the spirochaetes in routine paraffin sections.** *J. Lab. & Clin. Med.*, **25**, 204–10. 1939.

The following technic is proposed: Dehydrate tissue blocks in graded alcohols, clear in xylol, embed in paraffin, cut 9–10 μ thick and place in abs. alcohol (2 changes). Place sections in the following solution for 1–1½ min.: uranium nitrate (4% in abs. alcohol), 20 cc.; gum mastic (25% in abs. alcohol), 40–50 cc.; abs. alcohol, 20–30 cc. Wash in 3 changes of dist. water. Place in 0.1% aq. $AgNO_3$ for 1.0–1½ hr. at 100° C. Dehydrate in graded alcohols. Place in 10–12.5% gum mastic (in abs. alcohol) for 5 min. Wash 3 times in dist. water. Reduce for 5 min. in 5% hydroquinone (hydroquinone, 10 g.; 12.5% alcoholic gum mastic, 1 cc.; dist. water, 200 cc.) Counterstain, if desired, with hematoxylin and eosin, cresyl violet, or fuchsin. Dehydrate, clear and mount. The method is simple, rapid and free from precipitates.—*John T. Myers.*

TAYLOR, DEAN M. **A study of procedures for detection of coliform organisms in Minnesota drinking water.** *J. Amer. Water Works Assoc.*, **32**, 98–104. 1940.

In order to find a method of detecting coliform organisms in water, less laborious and time consuming than the *Standard Methods* "completed test", 1,534 samples of drinking water were tested using (1) that method, (2) brilliant green lactose bile and (3) fuchsin lactose broth. The second compared very favorably with the first and might advantageously replace it. For isolating coliform organisms, the alternative "completed test" of *Standard Methods*, employing brilliant green lactose bile, was preferable to the usual "completed test".—*Merritt N. Pope.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the April number of this Journal.

STAINS CERTIFIED MAR. 1, TO MAY 31, 1940*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Nigrosin WS	CNi–3	....	For negative staining of bacteria	Mar. 18, 1940
Chlorazol black E	NBc–1	....	As histological and cytological stain	Mar. 18, 1940
Eosin Y	CE–10	92%	As histological stain and as a constituent of blood stains	Apr. 1, 1940
Crystal violet	CC–10	90%	As histological, cytological, bacteriological stain, and in bacteriological media	Apr. 1, 1940
Carmine	LCa–2	....	As histological and cytological stain	Apr. 4, 1940
Carmine	NCa–7	....	As histological and cytological stain	Apr. 4, 1940
Alizarin red S	LAr–2	....	As stain for bone	Apr. 10, 1940
Congo red	NQ–8	87%	As histological counterstain	May 10, 1940
Nigrosin	NNi–7	....	For negative staining of bacteria	May 16, 1940
Eosin Y	NE–15	87%	As histological stain and as constituent of blood stains	May 23, 1940
Crystal violet	NC–21	90%	As histological, cytological, bacteriological stain, and in bacteriological media	May 24, 1940
Brilliant green	NBg–9	94%	For use in bacteriological media	May 31, 1940

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

STAIN TECHNOLOGY

VOLUME 15 OCTOBER, 1940 NUMBER 4

AN APPLICATION OF THE FROZEN SECTIONING TECHNIC FOR CUTTING SERIAL SECTIONS THRU THE BRAIN

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ABSTRACT.—The availability of CO_2 ice makes it practical to cut large blocks of cerebral tissue by the freezing method. If the tissue is first treated with 20–30% ethyl alcohol for sufficient time to secure uniform penetration of the alcohol (about 24 hours), formation of hard ice crystals can be controlled and serial sections 25–100 μ thick can be cut with negligible loss. The alcohol can be added to the fixative used for perfusion, or it can be added at any time later in the fixing process, or after fixation is completed. The sections are cemented to the slide and groups of slides are manipulated thru staining processes in glass trays. Ordinary cell and fiber stains give satisfactory results. The method is particularly useful for certain neurophysiological purposes such as defining the location of electrode tracks and lesions and certain types of retrogrades. The Prussian blue test for electrolytically deposited iron can be conveniently applied in conjunction with other stains, to determine the point at which a given action potential response was observed, if steel electrodes are used.

The determination of the extent of ablations or of the locus of electrode tracks and electrolytic lesions in the brain is a problem encountered in many studies of central nervous functions. For most of these purposes serial sections are either necessary or desirable. Three years use of the frozen section method has led to a procedure which is adequate for many purposes and which can be conducted with considerable economy of time.

The chief obstacle to cutting serial sections of cerebral tissue by the freezing method is the formation of hard and brittle ice crystals which fracture the sections as they are cut. If the tissue is first treated with a 20–30% solution of ethyl alcohol for about 24 hours this difficulty can be avoided. The time required depends on the size of the block; enough time must be allowed to permit uniform penetration of the

alcohol into the tissue. It does not appear to matter how the alcohol solution is used. Any fixation solution to which alcohol can be added may be made up with sufficient alcohol to make a 20–30% solution. Thus the brain can be sectioned, without washing, 12–48 hours after perfusion of the brain. The alcohol can be also added after fixation has begun or is complete; or, after fixation, the brain may be washed overnight and then put into 20–30% alcohol in water for 24 hours and sectioned. In any case, the material to be sectioned should be cut into blocks approximately one centimeter thick. It is sometimes desirable to strip off the pia and usually necessary to remove the extensive vascular tissue at the base of the brain. This can be done most safely the second day of fixation.

When ready for cutting, the tissue is placed on a CO_2 ice freezing block, which has been covered by a piece of wet blotting paper. The solid CO_2 ice is ground in a mortar to a conglomerate of small pieces of ice and snow and the ice chamber is filled with the mixture. For large blocks a little 95% alcohol can be put into the ice chamber with the CO_2 ice to hasten the initial freezing, but the speed of freezing must be increased with caution. Any open places where definite structural discontinuities exist are filled with water. A narrow ice bank must be similarly built up around the edges of thick blocks. It is usually necessary to build up the bank and open spaces by adding water drop by drop, as the block freezes, over a period of 10–15 minutes. As soon as the block is frozen thruout, most of the CO_2 ice is removed from the chamber.

While formation of hard and brittle ice crystals is chiefly controlled by the low concentration alcohol solution, some precautions are necessary in cutting the sections. It is necessary to avoid freezing the block too quickly and to avoid freezing it any harder than necessary. With each stroke of the knife the cut section is removed by means of a camel's hair brush kept wet with 50% alcohol, and it is necessary to paint any ice banks around the block and the colder portions of the block with 50% alcohol. This can be done in a second or two. The appearance of the surface, the force required to push the knife, and the sound the cutting produces are all guides to the estimation of the proper degree of hardness. A little experience teaches the correct judging of this part of the procedure. The essential thing is to keep the surface of the block near the freezing point; the sections are cut from a surface frozen only hard enough to cut cleanly.

A Bausch and Lomb clinical microtome with an 11 cm. paraffin knife is used in the writer's laboratory.[1] The angle of the knife with the block is not critical; it may vary

[1]Credit is due to Mr. J. I. Wexlin of the Bausch and Lomb Company for securing certain modifications of the frozen section microtome and for much helpful advice.

from 20° to 30°. The knife is usually set in a line perpendicular to the direction of motion. Tissue that has been fixed with acetic acid is cut by a razor blade clamped in a device which, in turn, is clamped in the regular knife clamps. There can be no objection to using this for any other type of work as we regularly cut with the knife fixed at right angles to the direction of motion. A freezing block is employed that is somewhat larger than that furnished with the microtome, but of the same general design. This block was cut out of a copper bar, but aluminum is probably to be preferred. There is no reason why other microtomes cannot be used, but the Bausch and Lomb model can be easily altered to give displacements of over 75 μ.

It is practical to mount sections 25–150 μ thick cut thru entire transverse frontal planes, or entire saggital planes of a cat's brain. There does not appear to be any reason why similar sections of larger brains cannot be cut with a correctly designed freezing block, but there has not yet been occasion to do so. As the sections are cut they are placed in order in an enameled pan or glass tray containing water or 30–50% alcohol and left there until the entire block is cut. Shrinkage and consequent curling of the sections can be kept at a minimum only by keeping them in a low concentration of alcohol or in water until they are mounted on the slides. It is helpful to lay glass strips in the pan which mark off the pan into alleys and thus keep the sections in order, in rows. Then they are mounted by placing the section or sections selected for a given slide in a dish of 50% alcohol. They are then floated onto the slide which has been previously coated with Mayer's egg albumin. Each section is smoothed out with a camel's hair brush and the slides are placed on a paraffin mounting tray or in an oven and dried at 40°–50° C. for 15 minutes to an hour.[2] The sections may also be pressed onto the slides with blotting paper, and the drying omitted. In any case, it is desirable to move the sections thru 70% alcohol to 80% alcohol to set the albumin before washing in water. Sections mounted from 50% or 70% alcohol adhere better than those mounted from water. Alkaline solutions should be avoided at each stage in the process of fixing and staining. If an alkaline fixative is used, the block can be made slightly acid by adding a little acetic acid sometime before cutting or the alcohol solution in which the sections are placed can be neutralized by addition of acetic acid. Even the laboratory water (pH 8) will detach the sections if they are washed in it for more than an hour. It is to be noted that silver processes usually involve an alkaline reducer We have found that hydroquinone formalin reducer as recommended by Bodian (1937) does not detach the sections. Amidol at an acid pH has not been tried; (see Davenport, *et al.* 1939).

Gelatin in 2% solution is a satisfactory adhesive if all subsequent procedures are carried out at room temperature.

[2]The author is indebted to Dr. H. W. Magoun for this and several other suggestions incorporated into the procedure.

The mounting is one of the least satisfactory processes in the method, for stray bits of choroid plexus and overlapping edges of cortical gyri are sometimes not seen before the section is stained.

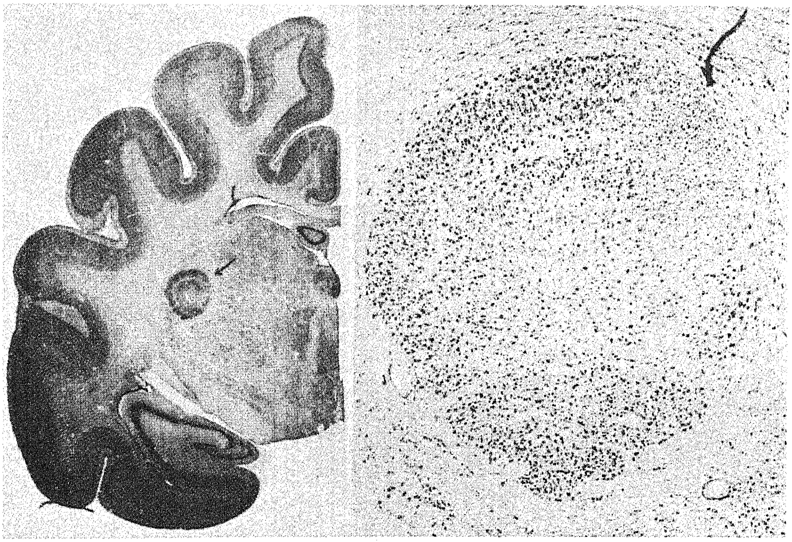
After the series has been mounted the slides are placed in glass trays and dehydrated thru a graded series of alcohol, placed in two changes of clove oil, ether-chloroform or xylene (preference in order named, but we usually use ether-chloroform) for approximately 15 minutes. After this treatment they are run down to water. Thionin (National Aniline and Chemical Company, Inc.), cresyl violet (suggested by Tress and Tress, 1935), toluidin blue (Coleman and Bell Company), and Weil's stains have given satisfactory results. For routine electrode placement sections, it is desirable to keep the differentiation time at a minimum. We have found that this can be done by washing the sections in distilled water 10–30 minutes after the above clearing process, then staining them 15–30 minutes at 50° C. in a saturated solution (less than 0.1%) made up in Sörenson's phosphate buffer calculated for a pH of 5.9. We concede that there is little justification for using this buffer because of the precipitating action of the phosphates. Critical illumination is of considerable aid in using these sections; see Carlson (1935).

There is no difficulty in cutting these sections serially with negligible loss thru a given block. We have not attempted to cut blocks thicker than 12 mm. This method is particularly well adapted for use in conjunction with a Horsley-Clark type of stereotaxic instrument, in which case the animal is perfused with fixatives to be described below, and the brain immediately blocked by partial cuts made by a sharp knife clamped to the needle carriage. These cuts overlap so that the brain is blocked in the plane of the electrode tracks. The animal is then removed from the stereotaxic instrument and the brain excised. When the brain is hardened the cuts are completed, making blocks the faces of which are approximately parallel with the electrode tracks, so that an entire needle track or a considerable portion of it is always seen in one section. For other purposes such as retrograde degeneration experiments it is desirable to outline the blocks similarly in the Horsley-Clark instrument, so that the nuclei desired can be included in a single block. It is, perhaps, worth noting that observation of the surface of blocks as they are cut affords a rather clear view of the gross orientation of electrode tracks.

Any of the usual fixatives should be satisfactory; those used by the writer are 10% formalin, 10% formalin in saline or Ringer's, 10% formalin in Ringer's with enough alcohol to bring its concentration up to 25%. The latter is necessary if sectioning is to be done the following day. It is possible that the fixation is better if the alcohol is not added to the perfusate, and the brain is kept in the formalin for two days, after which the alcohol can be added and the tissue cut a

day or two later. Fixatives causing appreciable swelling of the tissue are obviously undesirable for use in acute experiments in which an area of the calvarium has been removed. For this reason acetic acid fixatives are not entirely desirable for perfusion. We have occasionally used 20% formalin for the purpose of reducing swelling during perfusion with acid mixtures.

This is a very practical means of locating electrode placements, for the brain can be cut and necessary sections stained the day following the experiment.



A photographic reproduction of a transverse section (× 2.3) thru the lateral geniculate nucleus of the cat and a photomicrograph of the lateral geniculate in a contiguous section (× 21.0). The cat was perfused with the alcohol, formalin, Ringer's combination and the fixation was completed in same. The sections were cut at 75 μ, and stained with 0.5% thionin (unbuffered water solution). The retrograde lesion, indicated by arrows, resulted from a small striate cortex lesion made 31 days before the animal was killed.

The Prussian blue test (Adrian and Moruzzi, 1939) for inorganic iron can be introduced in a very convenient way to locate with precision the point at which steel electrodes recorded a given action potential response. Immediately after the observations have been made a current of 10–20 microamperes is passed for 5–10 seconds thru the electrode by putting the positive side of a 3–4.5 volt battery to the electrode connection. The negative pole is connected to a diffuse ground. At the conclusion of the experiment the animal is perfused with a solution consisting of 10% formalin, and 1% potassium ferrocyanide, in Ringer's solution. After the brain is blocked as described above, the animal is removed from the Horsley-Clark in-

strument and the brain is excised and put into the same fixing solution plus enough acetic acid to make a 2% solution, and enough alcohol to make a 20% solution. A day or two later the brain can be sectioned without washing. The electrolytic deposits of iron are a well defined green, usually visible to the naked eye. Thionin in phosphate buffered solution used as described above provides an adequate stain for nuclear groups and contrasts well with the green of the iron; see Talbot (1940). It is also adequate for defining the extents of many types of experimental lesions (Bard, 1940), and is useful for many types of retrograde degeneration experiments. (Figs. 1 and 2.) It is to be noted that thicker sections prepared by this method contain no more tissue than ones 70% as thick cut from blocks imbedded in celloidin or paraffin because of the small amount of shrinkage. This factor is also of advantage in checking electrode spacings.

ACKNOWLEDGMENTS

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COMBINED FIXING, STAINING AND MOUNTING MEDIA

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ABSTRACT.—A number of non-volatile, water-soluble substances can be added to the usual aceto-carmine fixing fluids. These inert substances do not alter the fixation image and serve as mounting media when the volatile ingredients of the mixture evaporate. Formulae are given for solutions containing dextrin, dextrose, gelatin, pectin, sorbitol, and sucrose. Gum arabic can be incorporated in a formic-acid-carmine fixative. The limiting factor in the use of such mounting media in fixing fluids is the osmotic value they give the solution; with certain precautions, however, they can be used in place of the usual aceto-carmine treatment. The indices of refraction of these media are not as high as those of the natural balsams and the fixation images which the mixtures produce have the characteristic limitations of those secured by the aceto-carmine technic. Some of the natural balsams (Canada balsam, sandarac and Venetion turpentine) can also be incorporated in fixing fluids. These fixatives are able to hold balsam and water in solution together, and, as the volatile components of the mixtures evaporate, the fixed specimens remain in permanent balsam mounts. The addition of carmine to these fluids enables us to fix, stain, dehydrate, clear and mount a specimen in a single operation. These fixatives preserve more details of chromosome structure than the aceto-carmine fluids, but their use is more limited; and they can be substituted for the latter only with certain favorable material, e.g., pollen mother cells of Rhoeo and Tradescantia and salivary gland chromosomes of Chironomus. Some of the newer synthetic resins can be substituted for the natural balsams. Formulae are given for fixatives which contain Venetian turpentine, sandarac, Canada balsam and two synthetic resins.

Recently there has been a great increase in the use of cytological methods permitting microscopic examination of specimens immediately after fixation. Perhaps the most useful of these procedures has been to macerate or smear the specimens in 45% acetic acid saturated with carmine (Schneider, 1880), thus fixing, staining and mounting the material in a single operation. The addition of a trace of iron greatly intensifies the stain (Belling, 1921, 1926). This acetic-acid-carmine technic has proven especially valuable for investigation of meiotic divisions in pollen mother cells and for examining the giant

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chromosomes in the salivary glands of the Diptera. The preparations thus made, however, are not permanent; and further treatment is necessary to preserve them for future reference (McClintock, 1929; Steere, 1931; Buck, 1935; Marshall, 1936; Bridges, 1937; Hillary, 1938, 1939; Burrell, 1939). The addition to the fixing and staining media of various inert substances, which do not alter the fixation image and which serve as mounting media when the more volatile components of the mixture evaporate, greatly facilitates the making of durable mounts. The very simplicity of such a technic gives it a number of practical applications.

These aceto-carmine fixing fluids which contain water-soluble mounting media can be applied successfully to a great many different types of material; in fact, if a few elementary precautions are observed, they can be substituted for the usual aceto-carmine mixtures. This method of making "permanent" mounts, however, has certain inherent limitations and should not be used when it is desired to preserve the finer details of chromosome structure. The water-soluble media have a lower index of refraction than the balsams and, while preparations kept in a dry place have shown no signs of deterioration over a three-year period, they are quickly destroyed if they come into contact with water. The most serious fault, however, is in the fixation image, for many of the finer details of the chromosomes are destroyed by 45% acetic acid. In fact, much of the more careful work of today avoids the acetic acid fixation image by means of a "pre-fixation" technic or by fixing first with a mixture which is less destructive than acetic acid, the aceto-carmine being retained in the technic primarily as a stain (Nebel 1939).

At present the balsams are by far the most satisfactory mounting media. Altho specimens have to be dehydrated and cleared before they can be impregnated with balsam, such preparations are used today almost exclusively. The studies herein reported, however, have shown it to be possible to make fixing fluids in which a balsam can be dissolved directly. The further addition of a dye produces a fluid which enables us to fix, stain, dehydrate, clear and mount the specimen in a single operation and thus simplifies greatly the making of permanent microscopic slides. As these fixatives preserve more detail than does 45% acetic-acid, and as the fixation images can be readily modified in a number of directions, preparations thus made have none of the disadvantages of those mounted in water-soluble substances. While this technic is not suited for preparing all types of material for cytological examination (it cannot be substituted generally for the aceto-carmine technic), it preserves specimens for which

it is fitted about as well as any of the more elaborate procedures.

Several of the newer synthetic resins can also be used as mounting media. They can be incorporated in fixing and staining solutions and treated precisely as the natural balsams. While none of those thus far investigated shows any particular advantages over the natural products, it is possible that some may be synthesized which will have optical properties and solubilities rendering them more satisfactory as mounting media.

I. Water-soluble Mounting Media

General Considerations. The more valuable water-soluble mounting media have been, with few exceptions, mixtures of two or more non-volatile substances. Farrant's solution (gum arabic, glycerin and water) and glycerin jelly (gelatin, glycerin and water) are typical of many such media. At least one component of each mixture must become an amorphous transparent solid on drying; and as these solids are generally too hard and brittle for permanent microscopic preparations, the practice has been to give them the proper consistency by adding to them a certain amount of glycerin. This has introduced the further complication, that when enough glycerin has been added, the fluids acquire such an osmotic value that they plasmolyze many of the more delicate specimens. Even when mixed with rapidly penetrating fixatives the glycerin may cause some shrinkage or prevent that swelling of the nuclear elements which has proved to be such a valuable attribute of many fixation images.

Indeed, the osmotic value of the non-volatile components limits very definitely the amount which can be incorporated in aceto-carmine fluids. On the other hand, these components must have a sufficient volume to occupy the space under the coverglass as the mount dries. Obviously, there will be a greater margin of safety if the substances which serve as mounting media have a relatively low osmotic value per unit volume. It is also necessary that the preparations dry out evenly and this demands that they be hygroscopic enough to pull water out from under the center of the coverglass to the edge where it can evaporate. For example, when gelatin is added to aceto-carmine, it solidifies very quickly around the periphery of the coverglass and seals off the rest of the mount from the air. The result is that the mount remains liquid for several weeks, and the subsequent uneven drying makes it worthless.

There are a number of solids which can be profitably incorporated in the aceto-carmine mixtures to form the basis of a mounting medium. Those investigated by the writer were dextrin, dextrose, sucrose, gelatin, gum arabic and pectin. These acquire a suitable

consistency, however, only when they are combined with some non-volatile water-soluble liquid. The liquids tested were glycerin, sorbitol (a hexa-basic alcohol) and gluconic acid. Glycerin was ultimately discarded in favor of sorbitol[1] for the latter has a larger molecule than glycerin and consequently has a lower osmotic value per unit volume. It also has a higher index of refraction and changes less under varying conditions of humidity. Gluconic acid apparently has no especial advantages.

It should be emphasized that the formulae here given are in no way definitive. They are merely samples of mixtures which have proved useful in mounting specimens of the pollen mother cells of *Rhoeo* and of several species of *Tradescantia*. They should be modified, of course, to suit whatever material is being investigated.

Dextrin. The dextrin should be c.p. and free from starch. The following formula is suitable for the pollen mother cells of *Tradescantia:*

Dextrin	10 g.
Sorbitol	10 cc.
Acetic acid	50 cc.
Water	60 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation[2]

The dextrin should first be dissolved in the water and the other components added in the order given. It is best to add the carmine after the ferric nitrate has been dissolved. The mixture should be brought to a boil and filtered.

Gelatin. If 10 g. of gelatin are mixed with 100 cc. of Belling's aceto-carmine (the gelatin should be dissolved in the water, the other components added and the whole boiled for 30 seconds and filtered), a liquid mounting medium is secured which automatically becomes sealed as the gelatin solidifies around the periphery of the coverglass. Such preparations dry out very slowly and may remain liquid for several weeks, the gelatin[3] furnishing an excellent, if temporary, seal.

For more lasting preparations the gelatin must be combined with some substance which insures that the entire mount will harden slowly. In the past, glycerin has been used for this purpose, and

[1]A satisfactory brand of sorbitol is sold by the Glyco Products Company, Inc., under the trade name of *Yumidol.*

[2]To saturate this and the following solutions with carmine, it is advisable to add 0.5 g. of the dye to each formula. This is somewhat in excess of the amount that will go into solution; but in the case of the venetian turpentine combination (page 147), it is a good safeguard to have some undissolved carmine at the bottom of the container.

[3]The gelatin is altered, of course, when it is boiled in acetic acid. It is not necessary for our purpose to identify the reactions.

glycerin may be incorporated in the aceto-carmine fluids. It is not recommended, however, for its osmotic value is too great in proportion to its volume. Parenthetically, it may be noted that the gelatin mounting media described below do not liquefy or soften in hot weather, and in this respect they are superior to the well-known glycerin-jelly. They have one defect, however, which need not be serious—small crystals are formed at the periphery. It should be emphasized that the exact proportions of the ingredients in these many-purpose fluids are to be modified to suit each specimen. The following formula may be useful as a point of departure for these necessary modifications:

Gelatin	10 g.
Sorbitol	10 cc.
Acetic acid	50 cc.
Water	60 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

Ten grams of sucrose can be substituted for the sorbitol in the above, altho when this is done the mixture should be brought to a boil, but the excess carmine should not be filtered out. The undissolved carmine which remains insures that the solution will remain saturated, for otherwise the color slowly fades. If dextrose is mixed with gelatin, the mounting medium becomes too hard and brittle for practical uses. Gluconic acid and gelatin can be combined as follows:

Gelatin	10 g.
Gluconic acid	15 g.
Acetic acid	45 cc.
Water	55 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

When gluconic acid is used, undissolved carmine should likewise remain in the solution. Even then the preparation may fade.

Gum Arabic, Gum Acacia. Gum arabic is soluble in 45% acetic acid but insoluble in stronger concentrations. Consequently, when it is added to aceto-carmine fixatives, the preparations become opaque when they dry in such a way that the water evaporates faster than the acid. On the other hand, the mount remains perfectly transparent if the acid evaporates faster than the water. Opaque mounts are avoided entirely if an acid more volatile than acetic is used. Pianese (1892) described a formic-acid-carmine combination which gives results in many ways more precise than the usual aceto-carmine fixatives, the chromatin being preserved with a sharper outline. As formic acid is also an aldehyde, it must never be mixed with gelatin.

The following is one of the most useful mixtures:

Gum arabic	10 g.
Sorbitol	10 cc.
Formic acid (87%)	41 cc.
Water	65 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

If small bubbles appear around the periphery of the coverglass, the amount of sorbitol in the mixture should be increased slightly and the amount of gum arabic decreased. The gum arabic can be most easily incorporated in the mixture if first pulverized in a mortar and dissolved in the water.

Pectin. Pectin[4] forms a gel in the presence of sugar and an acid. However, enough pectin cannot be added to aceto-carmine to serve as a mounting medium; other non-volatile substances must supply the volume. The advantage of using pectin lies in the fact that the preparation becomes solid as soon as it is made, altho it does not become really hard until it dries. There are a number of commercial brands of pectin available, the easiest to use being "Certo." Karo corn syrup (Patrick, 1936) is a satisfactory source of dextrose. The following mixture forms a usable mounting medium.

Certo (pectin)	10 cc.
Karo (dextrose)	10 cc.
Sorbitol	5 cc.
Acetic acid	55 cc.
Water	55 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

The mixture should be filtered and the excess carmine removed before the Certo is incorporated.

Directions. Aceto-carmine fixing fluids which contain mounting media are more viscous than those which do not. This introduces a complication in the smear technic, for delicate cells may be distorted in such a liquid by a too vigorous smearing. If pollen mother cells are to be fixed, the whole anther should be covered by the fluid for from 30 to 60 seconds before it is crushed. The cells should be examined immediately after they are extruded from the anther. They should not be shrunken; if they are, the mixture should be diluted with Belling's aceto-carmine. Very delicate cells can be more safely smeared in Belling's solution and then mounted in the

[4]For a detailed description of the pectin gels, see the contributions of L. W. Tarr, L. L. Baker and P. B. Myers in the Delaware Agr. Exp. Sta. Bulls., 136–187. 1924–1934.

mixture. It is important, however, not to mistake the absence of the customary swelling of the cells for a shrinkage. This swelling, which is such a valuable attribute of the aceto-carmine technic, occurs only when the preparation is flamed. The mount should be heated almost to the boiling point to secure the optimum swelling and staining. The amount of solution to be used depends upon the size of the cover glass and the desired thickness of the finished preparation; three or four drops of the solution are generally sufficient. Flexible number 0 cover glasses adhere to the media better than do the thicker types. If some of the medium is extruded around the edge of the cover glass, it should not be removed for it greatly strengthens the mount. For the most satisfactory results, it is necessary to modify and adapt the technic for each type of specimen to be mounted.

II. Fat-soluble Mounting Media

General considerations. To the best of the writer's knowledge, the technic of dissolving a balsam in a fixative has not been used hitherto. Under the circumstances it may be well to consider the principles involved in such a procedure in addition to the usual *ad hoc* directions and detailed recipes. Dissolving a balsam in a non-aqueous fixing fluid which gives an acceptable image offers no real difficulty. The only additional requirement is that the mixture be able to dissolve the water which is in the specimen itself without losing the balsam by precipitation. The addition of a reliable stain, however, introduces complications; in fact, hematoxylin and auramin were the only dyes found which stain the chromatin differentially when incorporated in these mixtures. Carmine[5] was too insoluble to be used. The intensity of the hematoxylin and auramin staining, however, could not be controlled accurately, and consequently the definition was generally poor. Another disturbing factor, which tended to make the staining still more erratic, was the alteration of the staining properties of the solution on standing.

The addition of water to the fixing fluids resulted in two marked improvements: (1) it enabled them to dissolve carmine, a dye easy to control, and (2) it increased greatly the control of the fixation images. As far as chromosome structure is concerned, the image may be altered in any one of a number of directions by changing the proportions of the ingredients; in fact, almost any of the usual fixation

[5]Carmine, of course, can be used in non-aqueous fixatives. In Carnoy's, for example, enough can be dissolved to stain the chromatin faintly. If methyl alcohol be substituted for the ethyl alcohol in the formula, a brilliant stain results. The fixation image, however, has little to recommend it and the mixture is not stable, the carmine being precipitated after a few days.

images are made available. On the other hand, the addition of water introduced another problem: it was necessary to hold the water and balsam together in a single solution, and this involved principles not generally met with in cytology.

Water and an oil can be made miscible by adding to them enough of a third liquid in which they are individually soluble. For example, if 10 cc. of xylene and 10 cc. of water are placed together in a graduate they will separate into two layers. If ethyl alcohol is added slowly, it will be partitioned between the xylene and water until 40 cc. has been added, whereupon it pulls the xylene and water into a single clear solution. In like manner it is necessary, if this technic is to prove practical, to hold both balsam and water in a single solution by reagents which, under these conditions, give a satisfactory fixation image.

The common reagents available in most laboratories of cytology differ greatly in their ability to hold water and an oil in solution simultaneously as is shown in the following table. The amounts are all expressed in round numbers, for the experiments were performed at laboratory temperatures and especially purified reagents were not employed.

TABLE 1. AMOUNT OF REAGENT REQUIRED TO HOLD WATER AND CERTAIN OILS IN SOLUTION SIMULTANEOUSLY

	10 cc. water and 10 cc. xylene	10 cc. water and 10 cc. oleic acid	10 cc. water and 10 cc. clove oil
Methyl Alcohol	90 cc.	80 cc.	70 cc.
Acetic Acid	60 cc.	115 cc.	30 cc.
Dioxan	60 cc.	50 cc.	25 cc.
Acetone	50 cc.	40 cc.	30 cc.
Phenol	50 cc.	35 cc.	25 cc.
Ethyl Alcohol	40 cc.	20 cc.	20 cc.
Tert.-Butyl Alcohol	30 cc.	30 cc.	25 cc.
Propionic Acid	30 cc.	40 cc.	20 cc.
Ethyl Alcohol —2 pts. n-Butyl Alcohol—1 pt.	30 cc.	25 cc.	20 cc.
Phenol —1 pt. Acetic Acid —1 pt.	30 cc.	20 cc.	15 cc.
Phenol —11 pts. Propionic Acid— 7 " Acetic Acid — 3 "	21 cc.	15 cc.	19 cc.

The reagents which are more effective in holding xylene and water in solution together are in general more effective in holding oleic acid and clove oil in solution with water. The table reveals, however, some marked exceptions. It is important to note that certain mixtures of the reagents are much more effective than any of the reagents used singly. For example, 60 cc. of acetic acid or 50 cc. of phenol are required to hold the xylene and water in solution, but when they are mixed in equal proportions only 30 cc. of the combination are needed. The mixture of phenol, propionic acid and acetic acid is the most effective yet found.[6] This mixture can be used as the basis of a fixing fluid.

Fat-soluble mounting media differ greatly in their tolerance for water. Thus, gum mastic and gum damar proved so intolerant that they could not be incorporated in any practical fixative. On the other hand, Canada balsam, sandarac, and Venetian turpentine were employed successfully; but inasmuch as Venetian turpentine provides a much greater margin of safety than the other two media, it alone is recommended. Formulae containing Canada balsam and sandarac are included only for completeness.

Venetian Turpentine. The proportion of the several ingredients in the following formula is determined by three considerations: (1) The Venetian turpentine and water must be held in a clear solution which does not become cloudy on drying; (2) a sufficient quantity of carmine must be soluble for staining; and (3) the fixation image must be acceptable.

Venetian turpentine	20 cc.
Phenol	55 cc.
Propionic acid	35 cc.
Acetic acid (glacial)	10 or 15 cc.
Water	25 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

To this it is sometimes necessary to add 5 drops of ethylenediamine. This formula can be put together in a graduated cylinder as follows: The propionic acid is first poured in and then the viscous Venetian turpentine is thoroly mixed with it. Next is added the phenol; either as loose crystals or a liquid. [If liquid phenol (88%)

[6]When phenol, propionic acid and acetic acid are added slowly to the xylene and water in the proportions indicated, the mixture of the five liquids emulsifies readily. When barely enough of the reagents has been added to pull the xylene and water into a single layer, the solution is clear in transmitted light, but opalescent in reflected light, indicating that some of the molecules are grouped in colloidal particles. The possibility is suggested that the molecules are definitely oriented with regard to each other and that the fluid possesses a certain structure.

is used, due allowance must be made for the water it contains.] The acetic acid comes next, and then the water. The ferric nitrate should be dissolved before any carmine is added. Under no circumstances should the mixture be heated. It may be filtered after 12 hours, altho some undissolved carmine in the bottom of the container does no harm. Special care should be taken not to allow the fluid to come into contact with the skin.

The proportions of the several ingredients in this formula have been derived empirically. They will doubtless have to be altered to suit the different specimens to which it is applied. Under the circumstances, it may be well to indicate briefly the role of each ingredient and to show the effects of changing its concentration. As the part played by Venetian turpentine is relatively passive, its concentration may be held constant and the variations limited to the other components.

The phenol ties the Venetian turpentine into the fluid, altho propionic acid may be substituted for much of it without causing any precipitation. A decrease in the amount of phenol decreases slightly the solubility of the carmine. The limiting factor in regard to phenol is the fixation image, as this is controlled by a very complex relationship between the phenol, the acids and water.

The propionic acid may be safely held constant. It has the same fixing properties as acetic acid, but it holds the fat-soluble components in solution better. It can take the place of some of the phenol, but nothing is gained by making the substitution.

It is perhaps more convenient to vary the acetic acid than any other component. This acid ties the water into the mixture. When the fluid is first made, 10 cc. of the acid is sufficient. On standing, however, the fluid alters somewhat and consequently a slight change occurs in its fixing properties. The original fixing properties can be restored by the addition of 5 cc. of acetic acid[7]. It may be noted parenthetically that the fixation image of most of the meiotic phases remains constant even if the proportions of the several ingredients are altered as much as 50%. The first meiotic metaphase, however, is very sensitive to such changes and it is possible, by keeping the phenol and water constant and varying the acetic acid, to secure a number of the more popular images. The proportions given here preserve the chromatic elements of the chromosomes of *Tradescantia* as spiralized chromonemata. This choice of fixation image is, of

[7]Even after standing six months the characteristic image of the fresh solution can be restored by adding another 5 cc. of acetic acid.

course, somewhat arbitrary, altho when this image is chosen, homologous chromosomes in different cells show less individual variation than they do when they are fixed as parallel threads, or, at the other extreme, as swollen, heavily staining bodies with a central row of vacuoles.

The water controls the amount of carmine which the solution dissolves. The valuable swelling of the nuclear elements characteristic of the aceto-carmine technic also depends upon the presence of of water.

The amount of iron mordant to be incorporated must be accurately determined. The best results for routine use are obtained by using either 0.5 g. of ferric nitrate or 0.2 g. of ferric chloride. If less iron is used, the stain is not sufficiently intense; if more is used, the iron tends to "salt out" the water so that the mount may become opaque on standing. This separation of water from the other ingredients as the mount hardens can be prevented by adding ethylene-diamine, which, by forming a complex cation with the iron, prevents the "salting out" effect. Thus, when it is desired to increase the staining capacity of the fluid, more iron can be included if at the same time ethylene-diamine is added. The quantities of each will have to be determined by experiments. Too much ethylene-diamine decreases the staining capacity of the mixture; too little allows it to cloud.

Canada balsam and sandarac. The following formula has given results comparable to those of the Venetian turpentine mixture. It is less tolerant of water, however, and consequently has a more limited usefulness.

Canada balsam or sandarac	10 g.
Oleic acid	10 cc.
Phenol	65 cc.
Propionic acid	40 cc.
Acetic acid (glacial)	15 cc.
Water	20 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

Directions and precautions. The limiting factor in the use of fixatives which contain a balsam in solution is the water which is in the specimen itself. If too much water is introduced, the preparation clouds on drying. The remedy is either to fix a smaller specimen or use more of the fixative. The amount of fixative also depends upon the thickness of the preparation and the size of the cover glass. In making smears of the anthers of *Tradescantia*, three drops are enough for a 22 × 40 mm. cover glass and four drops for one 24 × 50 mm.

The following method has been successful with pollen mother cells of this species. The anthers are placed uncrushed on a *dry* slide and covered by one drop of the fixative, which is allowed to penetrate for from 30–60 seconds. The anthers are then smeared and two more drops of the fixative added. After standing a few seconds, the preparation is ready for the cover glass. The preparation should *not* be flamed. The characteristic swelling occurs immediately on fixation, altho the stain becomes slightly more intense during the first 24 hours. This slide is permanent as soon as it dries. No special precautions are necessary in the dry air of a steam heated laboratory; the slide may simply be left to harden on a table. On a humid day in summer, however, or in the moist air of a seaside laboratory, the water evaporates relatively slowly, while the acids and phenol evaporate at their usual rate. If the water evaporates more slowly than the other volatile components, the mount becomes opaque. This can be prevented by drying the slide on a hot plate or in a desiccator. It can even be dried in a paraffin oven (60° C.) without injury.

Limitations. These fluids have been used successfully in making smear preparations of small root tips of *Zea Mays* and in preparing permanent mounts of salivary gland chromosomes of *Chironomus*. They are suited, of course, for fixing, staining and mounting the pollen mother cells of many different plants. For some material, however, they are quite unsuited; e.g., the pollen mother cells of *Oenothera* and *Gossypium*. Apparently any specimen which contains tannic acid, or any other compound which forms a precipitate with the iron mordant, cannot be treated successfully with this technic.

III. Synthetic Resins

Recent advances in the synthesis of new resins promise to provide mounting media superior to any now in use. Both water-soluble and fat-soluble resins are available, and it is possible that some of these, which the writer has not investigated, have just the properties which are needed. None of the water-soluble resins which were tested, however, proved stable in the concentration of acetic acid necessary for the aceto-carmine technic. On the other hand, two of the fat-soluble resins were stable in the mixture of phenol, propionic acid, and acetic acid, and as they were slightly more tolerant of water than Venetian turpentine, they might be preferred to the latter under certain conditions. This advantage, however, is slight, and as Venetian turpentine has a higher index of refraction and adheres better to glass, it should be used in preference to these where possible.

Duraplex V-240.[8] The formula for the mixture containing this resin is as follows:

Duraplex V-240	30 cc.
Phenol	60 cc.
Propionic acid	40 cc.
Acetic acid	15 cc.
Water	30 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

This is to be used precisely as the Venetian turpentine mixture.

NY-83-8. This synthetic resin is handled more easily as a 70% solution in methanol. It does not adhere to glass as well as Venetian turpentine. The following formula can be used:

Ny-83-8 (70% in methyl alcohol)	30 cc.
Phenol	50 cc.
Propionic acid	35 cc.
Acetic acid	20 cc.
Water	30 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

Lucite. Lucite is the trade name of a plastic made by E. I. du Pont De Nemours & Co. It is soluble in the mixture of phenol, propionic acid, acetic acid and water; and as it has a high index of refraction and adheres well to glass, it forms an excellent mounting medium. Two of its characteristics, however, render it unsuited for this technic. Mixtures which contain as little as 8% Lucite are so viscous that unless great care is taken, the cells of the specimen will be distorted when they are smeared in it. A more serious defect appears as the mount hardens. Fine droplets of water form (often temporarily) and any cells in contact with them become badly shrunken. The related mounting medium, isobutyl methacrylate, is even less tolerant of water than Lucite itself.

All grades of Lucite are soluble in the fixing mixture. Soft crystals (H7500) with type "D" plasticiser are easiest to handle. While passable slides can be made with the following recipe, it is not recommended.

Lucite	10 g.
Clove oil	10 cc.
Phenol	65 cc.
Propionic acid	45 cc.
Acetic acid	20 cc.
Water	20 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

[8]This is the trade name of a resin produced by *The Resinous Products and Chemical Co., Inc.*, of Philadelphia. The other resin designated Ny-83-8 is also their product.

Conclusions

Two types of mounting media which can be incorporated in fixing and staining solutions have been described. Those which are water-soluble can be added to the usual aceto-carmine fluids (gum arabic in formic-acid-carmine) and can be used wherever the usual aceto-carmine methods are suitable. The limiting factor is the osmotic value which the media give the solutions, so that sometimes special precautions are necessary. The swelling which is such a valuable consequence of acetic acid fixatives can generally be secured by heating the preparations to the boiling point. Slides made by this method have been kept in a dry place for three years and have shown no signs of disintegration. The fixation image has the characteristic limitations of that given by the aceto-carmine fluids.

Fat-soluble mounting media (Canada balsam, sandarac, Venetian turpentine) can be incorporated in a fixing and staining solution composed of phenol, propionic acid, acetic acid, and water. The fixation image given by this solution can be controlled more precisely than that of the aceto-carmine fluids. The limiting factor in the use of these fixatives is the water in the specimen itself. Sometimes special precautions are necessary in drying the preparations, but once dry they are as permanent as the usual balsam mounts. This technic cannot be used with material which contains tannin or other substances which precipitate the iron mordant. These fluids have been successfully employed on the pollen mother cells of *Rhoeo* and several species of *Tradescantia* and on the salivary gland chromosomes of *Chironomus*. They are not suited for preparing the pollen mother cells of *Gossypium* or *Oenothera*.

Synthetic resins can be incorporated in the mixture of phenol, propionic acid and acetic acid. One of them, Duraplex V–240, has a greater tolerance for water than has Venetian turpentine, altho it does not adhere to glass as well. Some of the newer resins will probably form better mounting media than any of the natural products now in use.

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THE SELECTIVE STAINING OF RED BLOOD CELLS

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ABSTRACT.—A method for the selective staining of red blood cells is described. Material is fixed in 10% neutral formalin in .85% NaCl and imbedded in paraffin or celloidin. Sections 6–10 μ are stained 1–5 minutes in chromotrope 2R. Basophilic and the less strongly acidophilic elements are decolorized with 5% phosphotungstic acid in 95% ethyl alcohol. Red blood cells and other strongly acidophilic elements that may be present in the preparation retain the chromotrope 2R. A counterstain of methyl blue may be used for staining the decolorized basophilic elements. As a result, erythrocytes are stained red by the chromotrope 2R, and basophilic elements blue, by the methyl blue. Less strongly acidophilic elements, having little affinity for either primary or secondary dye, are colorless or gray.

In a previous paper[1] the writer described a staining method for the differentiation of the acidophilic elements of tissue into two groups. Sections, appropriately fixed, were stained with erythrosin or other substituted fluorescein dye and differentiated in phosphotungstic acid. Following immersion of the sections in absolute ethyl alcohol, all tissue elements were decolorized with the exception of those strongly acidophilic. This group included erythrocytes, eosinophil granules, certain secretion granules, alpha cells of the pituitary, keratinized epithelia and Russell's bodies. The decolorized basophilic elements, collagen, reticulum, cartilage, bone, mucin, beta cells of the pituitary and basophil granules, were counterstained with anilin blue or light green. Less strongly acidophilic elements, including smooth muscle, striated muscle and the ground substance of most epithelia were characterized by their slight affinity for either primary or secondary dyes.

Since the publication of this paper, the technic has been simplified and a dye, chromotrope 2R, has been found superior to the fluorescein dyes for this procedure. The present technic is especially designed for the selective staining of red blood cells. The application of chromotrope 2R for staining the other above mentioned strongly acidophilic elements is under further investigation at this time.

[1]Crossmon, G. C. 1939. Separation of the acidophilic elements of the tissues into two groups. Anat. Rec., 73, 163–70.

The procedure is as follows:

1. Fix in 10% neutral formalin in 0.85% NaCl for 24–48 hours. Commercial formalin may be neutralized by the addition of marble chips a few days prior to fixation. Other fixatives may be employed but are not recommended for the preservation of red blood cells. Following fixation, material is washed in several changes of 70% ethyl alcohol to remove excess formalin.

2. Dehydrate completely, imbed in paraffin or celloidin, cut and mount as usual. It is important that sections be thin; with increasing thickness, decolorization in step 5 becomes more difficult. Six to 10 μ is suggested.

3. Stain sections in chromotrope 2R.

Chromotrope 2R (Grübler or National Aniline Co.)	0.25	g.
Distilled water	100	cc.
Glacial acetic acid	1	cc.

A suggested staining time is 1–5 minutes.

4. Rinse sections in 4 or 5 changes of distilled water. The last change of distilled water should show no trace of dye.

5. Transfer sections to 5% phosphotungstic acid in 95% ethyl alcohol. Agitate the slides occasionally while in this reagent. Preparations are left in phosphotungstic alcohol until as observed under the microscope all tissue elements are decolorized with the exception of red blood cells. The time varies with the thickness of the sections and the thoroness of the distilled water rinse in step 4. Basophilic elements are readily decolorized. Less strongly acidophilic elements, especially striated muscle, may require as long as 2–3 hours.

The specificity of the stain has been tested by leaving sections in phosphotungstic alcohol for a period of 36 hours without the slightest decolorization of red blood cells.

6. Transfer to methyl blue.

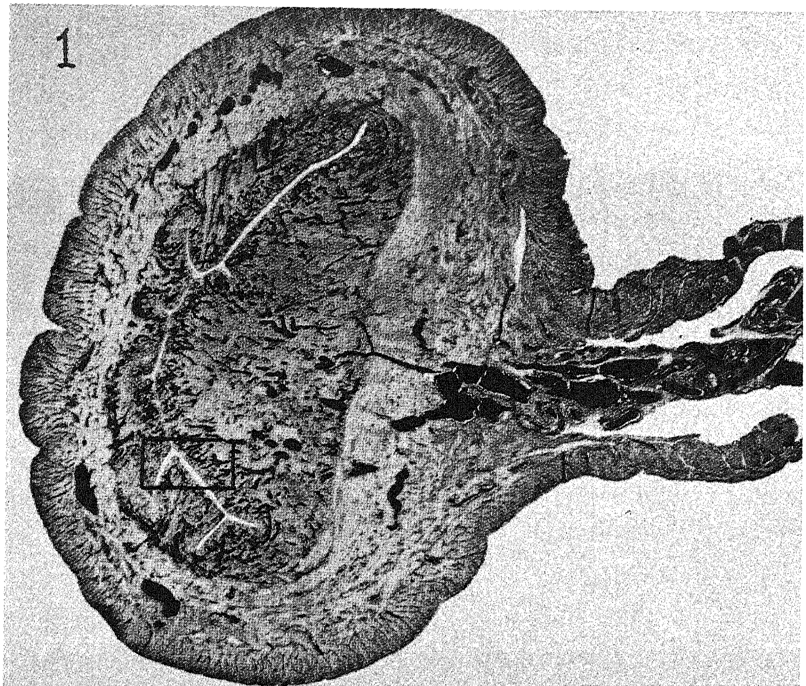
Methyl blue (National Aniline Co.)	0.5	g.
Distilled water	100	cc.
Glacial acetic acid	1	cc.

A suggested staining time is 2–5 minutes.

7. Rinse in distilled water.

8. Transfer to 2% glacial acetic acid for approximately 15–60 seconds. Sections should be examined microscopically at this time. As a result of this differentiation, erythrocytes should be red, less strongly acidophilic elements colorless or gray, and basophilic elements blue.

9. Pass thru 2 or 3 changes of absolute ethyl alcohol followed by 3 changes of xylol. Mount in balsam.



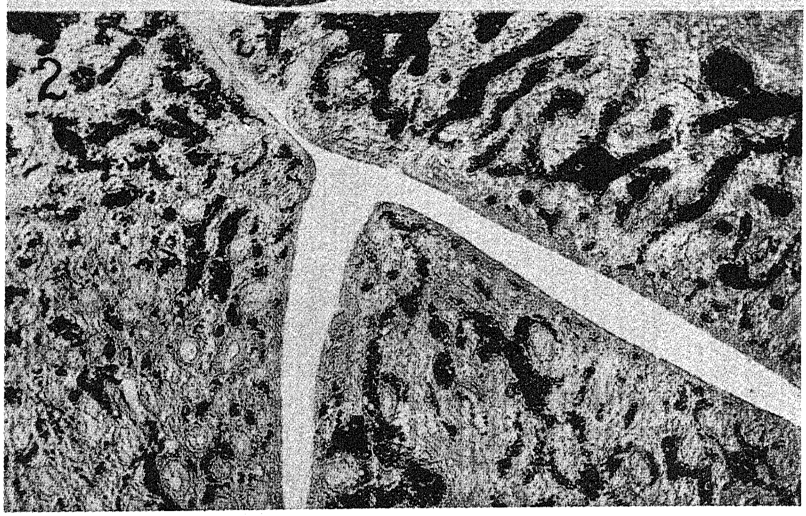


Figure 1. Photomicrograph of uterus of rabbit. Chromotrope 2R and methyl blue. × 10.

Figure 2. Photomicrograph of the area indicated within the rectangle in Figure 1. × 100.

Results and Discussion

Staining results are as given in step 8. The method is selective for red blood cells but not specific. Other strongly acidophilic elements, if present, retain the chromotrope 2R. If it is desired, the technic may be simplified by omitting the counterstain of methyl blue. In this case erythrocytes are stained red on a colorless background of the less strongly acidophilic and basophilic elements. A nuclear stain of freshly prepared Weigert's iron hematoxylin may be used as a preliminary to staining with chromotrope 2R.

Altho thick sections are not suggested, the writer has decolorized celloidin sections up to 25 μ. Such preparations are very striking and are suggestive of carmine-injected material for the demonstration of the circulation.

ACID FUCHSIN AS A CONNECTIVE TISSUE STAIN AFTER PHOSPHOMOLYBDOTUNGSTIC MORDANTING

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ABSTRACT.—**Certain acid fuchsins stain connective tissue deep red after phosphomolybdotungstic mordanting in a modified Masson procedure, others are entirely unsatisfactory for this purpose. Spectrophotometric examination gives no reliable criteria for separation of acid fuchsins satisfactory for this purpose from unsatisfactory ones. Sulphonation of basic fuchsin with 3.5 to 4 parts of 25–30% fuming H_2SO_4 to 1 part of dye gives a satisfactory product at temperatures as low as 65 to 70° C. in 30 minutes, while use of 5 to 7.5 parts of acid at this and at higher and lower temperatures gives unsatisfactory products. Satisfactory products may be produced with 15% fuming H_2SO_4 in similar quantities, and even with concentrated H_2SO_4, but some unconverted basic fuchsin remains with both and, with the latter, lower quantities give unsatisfactory products. Brief chemical studies indicate that oversulphonation may occur in the manufacture of acid fuchsin and that this is just as deleterious as undersulphonation.**

In the course of recent studies on trichrome staining (Lillie, 1938), it was noted that acid fuchsin (C. I. No. 692, lot NR–10) repeatedly failed to give a red connective tissue fiber stain after phosphomolybdic acid mordanting in a modified Masson technic. In this, acid fuchsin appeared to differ from the other commonly used diamino- and triamino-triphenylmethane sulphonic acid dyes such as light green SF (C. I. No. 670), fast green FCF, methyl blue[1] (C. I. No. 706), and anilin blue (C. I. No. 707). This discrepancy was quite disturbing from a theoretical point of view. Another old sample from Eimer and Amend also consistently failed to stain connective tissue.

Later two fresh lots of acid fuchsin, NR–11 and CR–6, were tested as fiber stains as a matter of routine confirmation of the previous results. Both of these samples repeatedly gave excellent red connective tissue staining by the following technic: Sections were first mordanted 1 minute in saturated alcoholic picric acid, washed 3 minutes in running water, stained 6 minutes in Weigert's acid iron chloride hematoxylin, rinsed in water, stained 4 minutes in 1% solution of naphthol green B (C. I. No. 5) in 1% acetic acid, rinsed in water, mordanted 1 minute in equal parts of 5% solutions of phos-

[1]See Lillie (1940).

phomolybdic and phosphotungstic acids, passed directly into a 2.5% solution in 2.5% acetic acid of the acid fuchsin being tested and stained 4 minutes, differentiated 1 minute in 1% aqueous acetic acid, passed thru alcohol, acetone, acetone and xylene aa, and xylene into salicylic acid balsam.

All four samples gave satisfactory connective tissue staining when mixed with picric acid by the van Gieson method.

The possibility was considered that a predominance of rosanilin or pararosanilin sulphonic acids might be responsible. To rule this out, 10 g. lots of an old Grübler sample of rosanilin sulphate, and of a 1936–7 sample of new fuchsin from Hartman Leddon Company, were sulphonated with 50 g. 25% fuming H_2SO_4 holding for 1 hour at 80–85° C. Both of the synthesized dyes gave good connective tissue staining.

Next the experiments of Scanlan, French and Holmes (1927) were repeated in part to ascertain whether the sulphonation conditions necessary to produce an acid fuchsin suitable for collagen staining after phosphomolybdotungstic mordanting are the same as those found best by them for acid fuchsins for van Gieson staining.

Our sulphonation technic is given here:

The fuming H_2SO_4 is weighed into a 150 cc. wide mouthed flask of general spherical form with flat bottom. The flask is clamped by its neck over a water or paraffin bath and a mechanical stirrer rigged in it. The bath is preheated to the desired lower temperature limit. Ten grams of finely ground basic fuchsin are then added gradually to the acid with the mechanical stirrer operating, avoiding boiling by slow addition of the dye. Then the sulphonation flask is immersed in the preheated bath and stirring continued for the designated length of time. When sulphonation is complete, a drop gives a clear red solution in water, which is decolorized by adding NaOH. Then the acid solution is poured into 350 cc. of cold water in a 2 liter beaker, and the flask is rinsed with 3 successive 50 cc. rinses of cold water into the same beaker. The diluted acid solution is then heated to boiling and neutralized with an amount of $CaCO_3$ slightly exceeding the quantity theoretically required by the amount of fuming H_2SO_4 used. For example, 40 g. of fuming sulphuric acid (30% free SO_3) requires approximately 43.6 g. $CaCO_3$; we used 45 g. The $CaCO_3$ is added gradually with constant stirring; the hot thick mixture is immediately poured into a large Buchner funnel and filtered into a vacuum flask. The large beaker is rinsed with successive washings to make a total of 500 cc. of boiling water and the washings are poured thru the precipitate. To convert the calcium salt to the sodium salt of acid fuchsin, 14 g. sodium bicarbonate is added, the solution is heated to boiling, set aside to cool over night, and the precipitate of $CaCO_3$ is filtered out. The solution is neutralized with 12–18 cc. of approximately 6 *N* HCl, testing with congo red paper. The deep red dye solution is evaporated to dryness over a steam bath. The yield is about 20 g. when 10 g. fuchsin and 40 g. fuming H_2SO_4 are used.

Ten-gram samples of a single lot of basic fuchsin were sulphonated for 15 minutes after the indicated temperature was reached, using at

each temperature 40 and 75 g. quantities of fuming H_2SO_4 containing about 25% free SO_3. The temperature ranges were 50–55° C., 65–70°, 80–85°, 95–100°, 110–115°, and 125–130°. Acid fuchsins NR–10 and NR–11 were used as negative and positive staining controls. The lots sulphonated with 40 g. fuming H_2SO_4 for 15 minutes at temperatures of 95–100° C. or higher were quite good, as was that sulphonated at 50–55° C. with 75 g. acid. The larger quantity of acid gave worthless products at 80–85° or higher temperatures, while with the 40 g. quantity of acid the lots prepared at the lower temperatures were all fairly good.

Following these tests, 10 g. lots of another batch of basic fuchsin were sulphonated with 40 and 50 g. quantities of 30% fuming H_2SO_4 at 65–70° C., holding for 15 and 30 minutes and one and two hours after the indicated temperatures were reached, making a total of 8 lots. Acid fuchsins NR–11 and E & A were used as positive and negative controls. This series indicated that the use of 50 g. of 30% fuming H_2SO_4 gave an inferior product and that with longer holding time, the product was poorer. With 40 g. acid the product was excellent and the optimum time appeared to be about 30 minutes. Consequently a larger batch was made with the same proportions, time and temperature. The product was excellent and colorimetrically apparently of higher dye content than any of the commercial samples.

To determine further the limitations on the proper quantities of basic fuchsin and acid for satisfactory sulphonation, another series of sulphonations was performed using the same temperature of 65°–70° C. and 30 minute time interval as previously.

In this experiment 12 g. portions of basic fuchsin were combined respectively with 40, 35, 30 and 25 g. portions of fuming H_2SO_4 containing 30% free SO_3; with 41.7, 36.2, 31.0 and 26.0 g. of a mixture of equal parts of the above fuming H_2SO_4 and of ordinary concentrated sulphuric acids; and with 42.5, 37.4, 32.0 and 26.7 g. of concentrated H_2SO_4.

The processes using the mixture of sulphuric and fuming sulphuric acids, and concentrated sulphuric acid alone yielded, at the stage of treatment with sodium bicarbonate, color precipitates which were soluble in hot water and in HCl and which reprecipitated quantitatively with picric acid solution. These precipitates appear to be unaltered basic fuchsin.

The final products were tested as usual in the Masson technic. Satisfactory products were obtained from sulphonation with fuming sulphuric acid using 40, 35 and 25 g. quantities, from the mixture of fuming and concentrated sulphuric acids in all quantities tried, and

from concentrated sulphuric acid in 42.5 and 37.4 g. quantities, each to 10 g. of basic fuchsin. The products were unsatisfactory with 30 g. fuming H_2SO_4 and with 32 and 26.7 g. concentrated H_2SO_4. (While H_2SO_4 containing only 15% free SO_3 appears to yield satisfactory acid fuchsins, the yield is reduced and some fuchsin escapes sulphonation.)

TABLE 1. COMPARISON OF VARIOUS ACID FUCHSINS IN THE VAN GIESON TECHNIC*

Sample Designation	Amount of acid fuchsin per 100 cc. picric				Rating by Masson Method
	25 mg.	50 mg.	100 mg.	200 mg.	
CR–6	+	++	+++	++	+++
NR–11	+	++	+++	++	++
NR–10	±	+	++	+	–
E & A	–	±	+	+	–
75–125–1	–	∓	±	+	+
75–110–1	∓	±	+	+	∓
75–95–1	–	∓	±	+	∓
75–80–1	±	±	+	++	∓
75–65–1	∓	±	±–+	+	+
75–50–1	±	+	++	++	+–++
40–125–1	∓	±	+	+–++	+–++
40–110–1	±	+	+	++	+–++
40–95–1	±	+	++	++	+–++
40–80–1	±	+	+	++	+
40–65–1	±	±–+	+	++	+
40–50–1	±	±	±–+	+	+
100/25–65–30	+	++	+++	++	+++

*The symbols in this table indicate:
+++ Excellent, ++ Very good, + Good, ± Fair, ∓ Poor, – No collagen staining.

As a measure of further control, many of these acid fuchsins were tested also by the van Gieson technic using various proportions of acid fuchsin as shown in Table 1. Fairly good correlation is shown but the van Gieson method does not give the sharp failures that become evident when the Masson method is applied.

Spectrophotometric examination of two samples CR–6 and NR–10, the one giving bright red connective tissue in the Masson technic, the other not, showed absorption maxima at 540 to 550 mμ in both.

TABLE 2. TRANSMISSION OF LIGHT BY ACID FUCHSINS CR–6 AND NR–10*

Wave Length in mμ	500	520	530	535	540	545	550	555	560	570	580	590	600	620	660	700	
CR–6	75	65	58	53	49	50	50	46	65		95		100	100	100	100	% Transmission
NR–10	75	65	56	60	49	50	49	54	60	75	95	100	100	100	100	100	% Transmission

*Note. Acknowledgment is made to Dr. M. I. Smith for these two examinations.

Believing that further spectrophotometric examinations might be of value, a number of spectrophotometrograms were prepared with the recording spectrophotometrograph of the Washington Biophysical Society and measured in detail. Table 3 gives a summary of these measurements. Characterizing NR–11, CR–6, 40–65–15, 40–65–30 and 100/25–65–30 as satisfactory acid fuchsins from the staining tests, and E & A, 75–125–1, 75–110–1 and 50–65–2 as unsatisfactory, no spectrophotometric character was found which would clearly separate these groups.

The comparatively high absorption percentage given by lot 50–65–2 (*50* g. fuming H_2SO_4 to 10 g. fuchsin, sulphonated at *65*–70° C. for *2* hours) at its absorption maximum when in aqueous solution at 5 γ per cc. indicates that this unsatisfactory sample has a comparatively high dye content. Hence it would seem that low dye content alone was not the reason for the poor staining. Doubling the acid fuchsin content of the 2.5% acetic acid solution used after the phosphomolybdotungstic mordant, while it did slightly improve the connective tissue staining, did not render it satisfactory. The E & A sample, the 75–125–1 and the 50–65–2 lots were thus tested. Except for CR–6, all satisfactory samples show a ratio of the absorption percentage at 530 mμ to that at 560 of 1.1 to 1 or higher, *but* on direct comparison of the stains CR–6 is better than NR–11. The median of

TABLE 3. VARIOUS SPECTROPHOTOMETRIC CONSTANTS OF ACID FUCHSINS

Sample	% Ab. 530 / % Ab. 560	% Ab. 520 / % Ab. 570	% Ab. at max, with 5γ per cc	λ max. Ab. %	λ 95% Ab.	Median λ 95% Ab.
Satisfactory NR–11	1.20	1.91	27.7	542–544	538–550	544
CR–6a	.917	1.15	35.9	547–548	542–556	549
CR–6b	.982	1.29	34.2	547–550	541–553	547
40–65–15	1.24	1.80	33.7	541–543	536–550	543
40–65–30	1.155	1.78	44.3	545	537–550	543.5
$\frac{100}{25}$–65–30	1.11	1.57	45.6	545–548	539–553	546
Unsatisfactory E & A a	.97	1.115	17.0	549–550	536–557	546.5
E & A b	1.00	1.07	27.7 at 10 γ	548–549	531–559	545
75–110–1	.98	1.37	17.2 at 20 γ	545	544–550	547
75–125–1	.923	1.06	18.2 at 20 γ	549–550	544–553	548.5
50–65–2	1.017	1.54	44.0	547	540–553	546.5
NR–10 (Visual)	1.10	1.40	About same as CR–6	545, 550	....	......

the zone showing over 95% of the maximum absorption percentage was between 545 and 549 with all the unsatisfactory samples, while the satisfactory samples ranged from 543 to 549, with 2 of the best at 546 and 548, the best and the poorest of the satisfactory samples at 543.5 and 544.

Chemical analyses of 5 samples were made by Senior Chemist Elias Elvove for nitrogen, total sulphur and free sulphate, sodium, calcium, magnesium and chlorine. After deducting calcium and magnesium, free sulphate and chlorine and enough sodium to satisfy the excess of anions over that required by the calcium and magnesium

and dividing the residues by the atomic weights, the following atomic proportions were obtained. (Table 4).

TABLE 4. ATOMIC PROPORTIONS OF N, S, AND NA IN ACID FUCHSINS

Sample	CR-6	100/25-65-30	75-125-1	50-65-2	E & A
N	3.00	3.00	3.00	3.00	3.00
S	3.36	2.52	5.80	3.54	1.50
Na	0.97	2.25	6.60	1.78	2.90

These results, while crude, indicate that the satisfactory samples 100/25-65-30 and CR-6 contain 2 to 3 sulphonic acid groups, that the marginal sample 50-65-2 probably contains 3 to 4 and that the 2 unsatisfactory samples 75-125-1 and E & A are markedly over-sulphonated and under-sulphonated respectively.

CONCLUSIONS

Acid fuchsins may be a satisfactory collagen stain after phosphomolybdic-phosphotungstic mordanting. If sulphonated with an excess of fuming H_2SO_4 or at too high a temperature, the product is unsatisfactory, probably on account of over-sulphonation. Too low temperature or insufficient amount and concentration of SO_3 also yield unsatisfactory products, probably on account of undersulphonation. The absorption spectra of satisfactory and unsatisfactory samples are similar.

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To Senior Chemist Elias Elvove for chemical estimations.

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THE ACETO-CARMINE METHOD FOR FRUIT MATERIAL

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Abstract.—It is not easy to make good aceto-carmine preparations of plants with small chromosomes at meiosis because the cytoplasm readily takes up the stain and this prevents a sharp differentiation. The staining reaction depends on the composition of the pre-fixative, the duration of fixation, strength of aceto-carmine and amount of iron used. These factors can be varied independently. Since not only species but their varieties differ markedly from one another in their behavior, the best results can be secured only after experiment with individual plants to discover the most suitable combination. Suitable combinations of these factors for some fruit plants are described. In general they demand (1) a weaker solution of aceto-carmine and more iron than has hitherto been used in the aceto-carmine technic, and (2) the introduction of iron and carmine into the pre-fixative. Iron acetate is added to a dilute solution of carmine in glacial acetic acid until the solution assumes a deep red color, without precipitation, and this solution is used as the acetic acid component of an acetic-alcohol pre-fixative. Anthers are colored purple by treatment with this fixative, but since it has only a mordanting effect they need to be smeared and stained in the ordinary way.

Plants with large chromosomes, such as *Tradescantia*, give excellent results with aceto-carmine; those with small chromosomes give inferior results owing to the readiness with which the cytoplasm takes up the stain and thus prevents adequate differentiation between chromosomes and cytoplasm.

Experience in this laboratory with the treatment of small chromosomes in various fruit species leads to the belief that what is needed is a radical modification of the fixation and mordanting methods used with aceto-carmine. With such modification these methods might be capable of giving as satisfactory results with small chromosomes as with large.

The staining reaction depends on four factors which can be varied independently: (1) constitution of the pre-fixative; (2) duration of fixation and storage; (3) strength of aceto-carmine solution; (4) amount of iron introduced.

Constitution of pre-fixative. Three parts of absolute alcohol to one

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of glacial acetic acid is as a rule quite a satisfactory pre-fixative for aceto-carmine. Variation in the proportion of alcohol and acetic acid alters the quality of fixation but does not appreciably change the staining reaction. Poor fixation, however, results in a poor staining reaction.

Improved staining results can be obtained by introducing both carmine and iron into the fixative. Glacial acetic acid is boiled with excess of carmine and filtered after cooling. A saturated solution of iron acetate in glacial acetic acid is added drop by drop until the carmine-acetic solution assumes a deep red color, without precipitation. This carmine-iron-acetic solution is used to make the 3:1 alcohol-acetic pre-fixative. Occasionally an undesirable precipitate forms in this fixative, either at once or some time after the anthers have been put in. This occurs when the original carmine-acetic solution is too concentrated. The procedure to adopt is to test small quantities of the solution for precipitation after diluting with various amounts of glacial acetic acid.

Anthers placed in carmine-iron-acetic-alcohol become purplish in color, but since the fixative has only a mordanting effect they need to be smeared and stained in the ordinary way. When the chromosomes have been pre-mordanted in this way, they enjoy the fullest advantages of differential staining. The fixative is suitable for all fruit plants except those which do not require much iron, e.g. *Ribes* (see schedule).

Duration of fixation and storage. Very soon after fixation (2–3 hours) satisfactory chromosome staining can be obtained, but the cytoplasm has a granular appearance (Fig. 1). It becomes clear after fixation for 12–24 hours (Figs. 3–5). If the material is stored either in the fixative or in 70% alcohol for a considerable time (1–2 months) it is again difficult to obtain a preparation in which the cytoplasm is clear (Fig. 2). Long storage, however, produces a sharp differentiation between spindle and cytoplasm, a fact which is sometimes useful for studying abnormal spindle development or the positions of the chromosomes relative to the spindle.[1]

To obtain a well-stained flattened cell it is essential to burst the pollen mother-cell wall without actually damaging the cell itself. In preparations made soon after fixation the cells are too delicate and are easily damaged, but on the other hand long fixation makes the cells too hard and it is not possible to burst the wall during the process of heating. The best time must be found by experiment.

[1]Darlington, C. D. and Thomas, P. T. 1937. The breakdown of cell division in a *Festuca-Lolium* deritative. Ann. Botany (London), n. s. 1, 747–61.

For most fruit plants, fixation for 12–24 hours gives the best results, but for *Ribes*, the cells are so delicate that they cannot be satisfactorily smeared for three days. Hardening can, however, be accelerated by transferring the material to Carnoy.

Strength of aceto-carmine solution and amount of iron introduced. La Cour[2] (1937) advocates dilution of the gentian violet stain for sharp and rapid differentiation of large-chromosome nuclei which take up the stain too readily, and it proves that by far the best root tip preparations of fruit plants also are obtained after dilution of the stain. Hitherto Belling's saturated solution of carmine in 45% acetic acid has been generally advocated in staining schedules. But in the case of aceto-carmine, as in that of gentian violet, a diluted solution makes for easier differentiation in the pollen mother-cells of fruit plants. Generally a one-third strength solution (diluted with 45% acetic acid) is suitable, but the best concentration varies for different plants. Altho iron has been added to the pre-fixative, it is still necessary to add considerably more than usual at the time of smearing. The amount needed again differs markedly for different plants. Pears and apples, for example, require a weak carmine solution and a large amount of iron; *Ribes* requires a weak carmine solution with only a trace of iron. The strength of the aceto-carmine and the amount of iron required are also dependent on the time of storage, weaker amounts being necessary after long storage.

The iron should be introduced during the process of smearing by teasing with iron needles or small scalpels, since aceto-carmine to which much iron has previously been added soon precipitates. The cytoplasm is less liable to become stained, however, if the time between smearing and heating is short; and with plants which demand a considerable amount of iron, it is necessary to dip the needle once or twice into a solution of iron acetate.

It is important to realize that one species or even variety may differ markedly from another in its reaction to aceto-carmine. Indeed, varietal differences may override any characteristic of the species and the importance of adapting the technic to the needs of the individual variety or clone must never be overlooked. In this respect the differences between diploids and triploids of the same species are very remarkable. My colleague, Mr. Raptopoulos, finds it impossible to obtain satisfactory staining for certain triploid cherries unless the acetic-alcohol pre-fixative described above is used, while for diploids and tetraploids the precaution is scarcely necessary.

[2]La Cour, L. 1937. Improvements in plant cytological technique. Botan. Rev., 5, 241–58.

Explanation of figures

All the photographs (except Fig. 4) were made from temporary preparations at a magnification of $\times$ 1000 and enlarged to $\times$ 3000 for reproduction. Except for Fig. 4 all the material was fixed in the mordant acetic-alcohol fixative described in this paper.

Fig. 1. *Prunus divaricata Moseri* (2n = 16). Bivalents and univalents. Stained in ½ strength carmine 5 hr. after fixation. Cytoplasm granular.

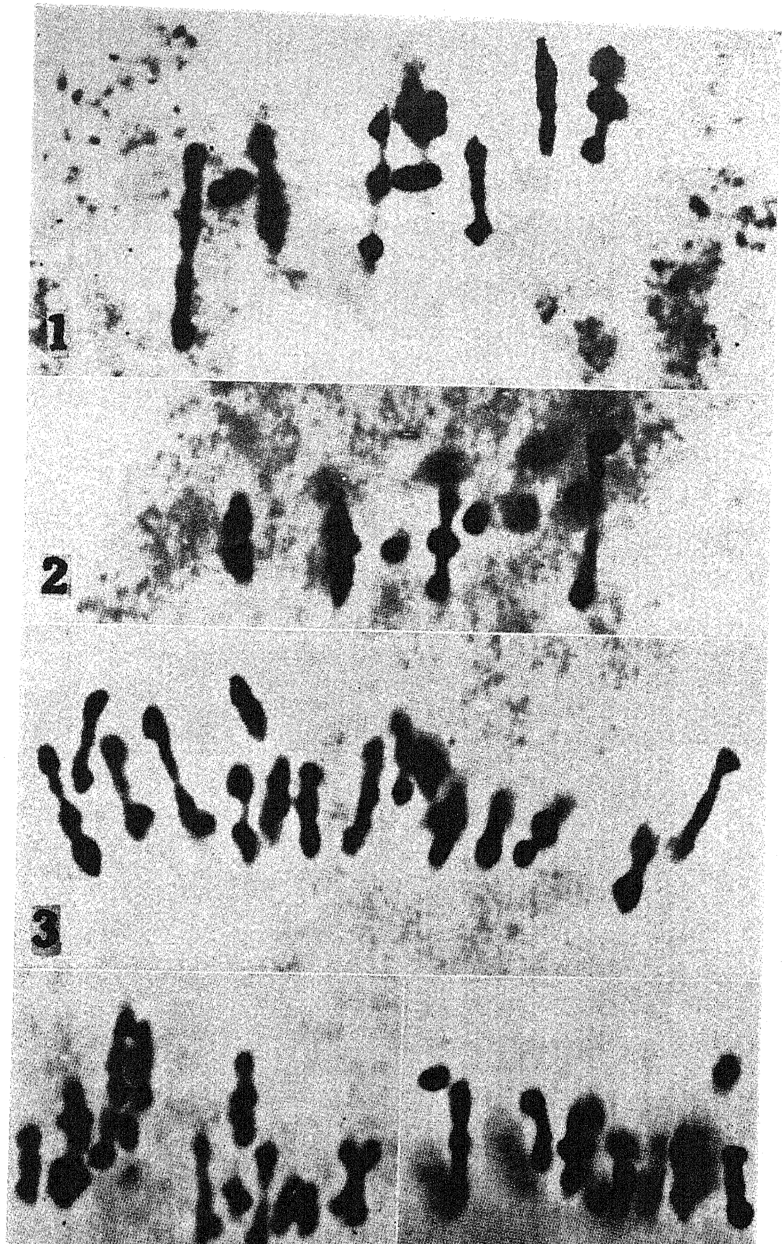
Fig. 2. *Prunus divaricata Moseri.* Bivalents and univalents. Stained in ¼ strength carmine 3 weeks after fixation. Cytoplasm not well differentiated.

Fig. 3. *Prunus spinosa* (2n = 32). 16 bivalents. Stained in ⅓ strength carmine 36 hr. after fixation.

Fig. 4. Duke cherry (2n = 32). Quadrivalents and bivalents. Stained in ⅓ strength carmine 24 hr. after fixation.

Fig. 5. *Pyro-cydonia* hybrid (2n = 34). Bivalents and univalents. Stained in ½ strength carmine 24 hr. after fixation.

Thanks are due to Dr. D. Eissler for the preparation from which Fig. 1 was made.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Critical studies should always be made on the temporary aceto-carmine slides, but permanent slides are useful for record purposes. All the photographs in the accompanying plate except Fig. 4 were made from temporary slides.

Experimental schedule. The following general schedule of aceto-carmine technic is suggested for small chromosomes:

(1) Fix the material in 1:3 acetic-alcohol prefixative and determine the optimum time after fixation when the P.M.C. wall can be burst without damaging the cell.

(2) By diluting Belling's saturated solution of carmine in 45% acetic acid (with 45% acetic acid) find the highest concentration which allows of clear cytoplasm.

(3) Determine the amount of iron which the particular plant needs for optimum chromosome staining without causing the cytoplasm to darken.

(4) If the amount of iron required is considerable, pre-fix in the mordant acetic-alcohol mixture described above.

NOTES ON TECHNIC

CHECKING AND ADJUSTING THE ALIGNMENT OF OBJECTIVES IN THE BINOCULAR DISSECTING MICROSCOPE.—This note concerns two methods found to be useful in keeping the binocular lens systems of this type of microscope in fair alignment; the first is for less refined work, the second is for more accurate work.

In the first method, the check is begun by swinging any one of the three sets of objectives (high, low or middle magnification) into position. Next, an index card is placed upon the dissecting stage. Using *one* eye at a time, the exact periphery of *each* field of vision is outlined by punching small holes into the card with a needle. No less than four holes should be made at the terminals of any two diameters which cross at right angles. When *binocular* vision is again used, the two fields as outlined by the two sets of holes in the card, should nearly coincide. If they do not, the binocular system is out of proper alignment, and the following adjustment is suggested: view the card thru the *left* ocular and see that a set of holes in the card exactly coincides with the periphery of that field; next, *without moving the card* (weight it down), remove the objective barrel and barely loosen the tiny screws which keep the *right* objective plate in position. Then, if the screws have been loosened just enough so that the *right* plate may be moved with slight fingering, and if binocular vision is resumed, this *right* plate may be moved so that the *right* field will finally agree with the *left* field, as denoted by the set of holes in the card. This process must be checked repeatedly by opening and closing the *right* eye and moving the objective plate until the two fields agree.

The second method, which is a more accurate one, involves a refined technic in which a cross-hair ocular or ocular eye piece is used. The method of checking the adjustment remains the same as above, or it may be modified by aligning the *point* of intersection in the cross-hair unit with a tiny *point* made on an immovable index card. In explanation, it may be noted that the diameters of the light diaphragms of the left and right ocular lenses might never be exactly the same. Thus the points of coincidence must of necessity be in the central axis of each lens system. This axis ought then to include the *point of intersection* of the cross-hair and the center of the microscopic field, as indicated by the meeting place of two diameters drawn at right angles upon the index card. In this better method the principle is the same as that suggested above. The *left* side of the binocu-

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lar system, containing the cross-hair unit is aligned, the ocular cross upon card cross, with exact central agreement. Next, the unit is moved to the *right* ocular position and the screws attending the *right* objective plate are loosened. This *right* plate is moved so that the crosses *again* exactly agree. Finally, the screws are carefully tightened.—CHARLES O. HATHAWAY, College of William and Mary, Williamsburg, Virginia, and FREDERICK F. FERGUSON, College of William and Mary—V. P. I., Norfolk, Virginia.

RESINS FOR SEALING GLYCERIN MOUNTS [WITH A NOTE ON THE USE OF CLARITE (NEVILLITE V)].—Among materials used for sealing glycerin, glycerin-jelly or glychrogel mounts to make permanent microscopic slides, resins comprise one group usually available in the biological laboratory. To use them, rings of diameter equal to that of the circular cover-slips selected are painted on microscope slides with a fine (No. 1–No. 3) camel's hair brush dipped in the resin; use of a turn-table insures regular rings. Just enough of the glycerin medium to occupy the space in the ring when the cover-slip is placed is applied with a pipette and, if necessary, is warmed to facilitate spreading. The correct amount can be determined only by practice and varies a little with various batches of glycerin jelly and glychrogel. If the preparation is warmed, it should be cooled first, otherwise sealed carefully with resin thinned with xylol or toluol. A second coat should be applied the following day.

In resins employed as seals, clearness and whiteness have been considered desiderata by the writers. Bell's cement, a proprietary seal, fulfills these requirements, but is expensive. Recently a great number of water-white resins have been synthesized of which a few have been tested by biologists as mounting media in place of Canada balsam and gum damar. Three of these are: Clarite (formerly called Nevillite V), developed by the Neville Co. of Pittsburgh, Penna.; Teglac 161, produced by Bakelite, Ltd. of London; and Distrene 80, by Honeywill and Stein, Ltd. of London. The writers have used Clarite (Nevillite V) both as a mounting medium and as a seal. Where great haste is not important, glycerin mounts are made using a store of previously "ringed" slides, and the seal is made using a solution of 60 parts of Clarite to 40 parts of toluol by weight. Where mounts need to be used within a few hours of preparation, the authors use "Cellobalm" made by dissolving 100 g. of ethyl cellulose in one liter of toluol with shaking at intervals over a day or two and then adding 200 g. of Canada balsam. The solution requires no filtration. A more viscous solution can be made by using smaller proportions of toluol.

With Clarite as a mounting medium some workers in our laboratory have found that the toluol solutions recommended by Groat[1] develop bubbles by contraction greater at one side than at others. It is noted, however, that this takes place when fresh slides are warmed for rapid drying. Where slides have dried at room temperature this difficulty is generally obviated. Kirkpatrick and Lendrum[2] in using the water-white Distrene 80 have added a plasticizer, tricresyl phosphate (7.5 cc. tricresyl phosphate, 40 cc. xylol, 10 g. Distrene 80) to prevent retraction and such a modification might be used with Clarite. The superiority of Clarite over Canada balsam and gum damar is most apparent in thick mounts such as whole mounts of large flukes (*Fasciola*), and *Amphioxus* where cells may reach 3 mm. in thickness.—J. L. MOHR and WM. WEHRLE,[3] University of California, Berkeley, Cal.

A NEW EMBEDDING SCHEDULE FOR INSECT CYTOLOGY.—Attention has recently been drawn to the fact that the carbolic acid and water technic simplifies the arduous task of sectioning yolky eggs. These difficulties have been experienced in a study of meiosis in the eggs of various sawfly species, and were successfully overcome by the use of *n*-butyl alcohol and phenol followed by the water treatment. Since much work is now being done on insect cytology and embryology, the time appears opportune to make the schedule available to others.

Petrunkevitch[4] first pointed out that the inclusion of phenol in a fixative gives "a peculiar elastic texture to the tissues, unlike anything produced by commonly used fixing fluids." Slifer and King,[5] however, found that sectioning after the use of one of Petrunkevitch's fixatives provides smooth, clean sections but that "Cytoplasmic details were badly distorted and the chromosomes were almost unrecognizable." By a process of elimination they found that the use of a standard cytological fixative followed by 24 hours soaking in a 4% solution of phenol in 80% ethyl alcohol resulted in equally fine sectioning qualities and at the same time preserved the normal microscopical features. The process of dehydration and embedding was accomplished by means of ethyl alcohol, carbol-xylol and paraffin. After the paraffin had set, the material was blocked

[1]Groat, R. A. Two new mounting media superior to Canada balsam and gum damar. Anat. Rec., **74**, 1. 1939.

[2]Kirkpatrick, J., and Lendrum, A. C. A mounting medium for microscopical preparations giving good preservation of colour. J. Path. & Bact., **49**, 592. 1939.

[3]Employed by the U. S. Works Progress Administration. Aid from this Administration (Official Project 65–108–113, Unit C–1) is hereby acknowledged.

[4]Petrunkevitch, A. 1933. New fixing fluids for general purposes. Science, **77**, 117–118.

[5]Slifer, E. H., and King, R. L. 1933. Grasshopper eggs and the paraffin method, Science, **78**, 366.

and the paraffin pared down until the end of the material was exposed and the whole was then soaked in water for 24 or more hours. Both the phenol and the water treatments are essential; the latter assists the paraffin in gliding over the microtome knife.

In certain species of sawfly the eggs are laid in pine and spruce needles which themselves are extremely tough and consequently add to the difficulty of sectioning the eggs. To prevent hardening during dehydration the *n*-butyl alcohol method of Zirkle was used but only with limited success. Distinctly better sections were obtained by means of the Slifer and King technic, but since the higher concentrations of ethyl alcohol and particularly the xylol have an inherent hardening effect, the following composite schedule was evolved: the results justify its recommendations.

Step	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Water	95	90	80	65	50	30	15	5	0	0	0
Ethyl alcohol	5	10	20	35	40	50	50	40	25	0	0
N-butyl alcohol	0	0	0	0	10	20	35	55	75	100	100
Phenol	0	0	0	0	0	0	4	0	0	4	4
Hours	½	½	½	1	1	1	24	1	1	1	—

After step 10 the material and butyl-phenol was placed on an equal amount of solid paraffin, transferred to the oven and changed to pure paraffin after 16 hours or so. Infiltration should be completed in a further four hours. The embedded material was trimmed and soaked as recommended by Slifer and King. Adhesion of the sections was improved by using slides thinly smeared with albumen. The albumen film should be dried and the ribbon floated on with the aid of 35% ethyl alcohol.

The step at which dehydration is commenced varies with the fixative used, e.g., after 2BD, step 1; after Kahle's fluid, step 4; and after Kahle's without water, step 6. Fixation in Kahle's without water, which gives an excellent stain with Feulgen, requires only two hours, so that the whole fixing and embedding process can be completed in approximately two days.—STANLEY G. SMITH, Department of Genetics, McGill University, Montreal.

DIOXAN FOR BLEACHING.—To bleach material fixed in osmic mixtures, either with or without acetic acid, immerse it in dioxan. Bulk material may require up to three days; sections a much shorter time. No additional treatment is required.—J. J. ASANA, Gujarat College, Ahmedabad, India.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

CROOKS, D. M. **The use of a translongitome in making and interpreting alternative transverse and longitudinal serial sections.** *Science*, 91, 150. 1940.

Translongitome is the name given to a new microtome attachment developed for the purpose of obtaining transverse and longitudinal sections from the same block. Such a two-plane cutting attachment can be used in a rotary or a sliding microtome. The sections come off in the form of a continuous ribbon in which they have been cut alternately crosswise and longitudinally. The device fastens into the microtome clamp, and consists fundamentally of a hinged sector capable of swinging thru an angle of 90° in a plane parallel to the knife edge. It is, of course, necessary to set the microtome to cut one half the thickness desired as each face of the block is cut on alternate strokes. The translongitome is manufactured under patent rights, and priced at $22.50.—*J. A. de Tomasi.*

ECKEL, EARL E. **Cover slip dispenser.** *J. Lab. & Clin. Med.*, 25, 882–3. 1940.

A machine is described which ejects cover slips one at a time in a position to be grasped by the edges without smearing the surfaces. The machine is composed of a plunger of correct thickness running on a horizontal plane thru a box containing cover slips, the edges of the box being raised sufficiently in the direction of the motion of the plunger to allow only one cover to be pushed out of the box. The box can be bent into shape from scraps of stainless steel or monel metal. The slit is adjustable for thickness of cover slip. The box and runway for the plunger is soldered to a heavy metal bar. The plunger is cut from the 0.006-inch blade of a thickness gauge. A pin is soldered to each end of the plunger and one to the back of the base to permit a rubber band to draw back the plunger.—*John T. Myers.*

JOHN, K. **Ein neues Universalgerät.** *Zts. wiss. Mikr.*, 56, 371. 1939.

A new universal microscope manufactured by Fuess of Berlin is described. It differs from the well known Leitz "Panphot" in placing the photographic portion of the apparatus to the left of the microscope on the same plane instead of superimposing the camera over the latter. An ingenious arrangement permits the light to be diverted either to the left for photomicrography or to the right for microprojection upon the drawing board. The illuminating unit and accessories are permanently built in and are suitable for opaque illumination as well as for work with the polariscope.—*J. M. Thuringer.*

MOSEBACH, GEORG. **A microprocess for the cryoscopic investigation of succulent tissues.** *Ber. deut. botan. Ges.*, 58, 29–40. 1940.

A microcryoscope is described by means of which osmotic concentrations may be determined on pieces of succulent tissue having a volume smaller than 1 cu. mm.—*Merritt N. Pope.*

PHOTOMICROGRAPHY

ARMITAGE, F. D. **An eyepiece camera for miniature negatives.** *The Microscope*, 4, 124–7. 1940.

The camera consists of two tubes and a focusing screen. The lower tube fits over a fiber washer that is placed over the eyepiece end of the body tube or draw-

tube, forming a light-tight joint. Between the two tubes of the camera are two diaphragms of fixed aperture; between these slides a metal plate serving as a shutter. At the top of the camera is a brass-bound wooden holder for the focusing screen or the double-sided plate-holder. The focusing screen is ruled in squares of ½ cm. sides; in its center is a cover-glass cemented with Canada Balsam so that a focusing lens can be used. The negatives (4.5 x 6 cm.) will bear considerable enlargement.—*C. E. Allen.*

AUER, A. **Die Verwendung des Rollfilms und die Messung der Belichtungszeit in der Mikrophotographie.** *Zts. wiss. Mikr.*, **56**, 259. 1939.

To determine exposure time for photomicrography the use of a small extinction type exposure meter such as the Zeiss Icon "Diaphot" is recommended. The aperture of this disc-shaped exposure meter is brought directly over the eye-piece. The gray wedge is rotated until the desirable microscopic detail just disappears. An exposure table is then made based upon known correct exposures and the corresponding meter reading for every lens combination used. For cameras with bellows, additional calculations must be made for various extensions.—*J. M. Thuringer.*

MICROTECHNIC IN GENERAL

BEYER, E. M. **Trichrome stain for astrocytes.** *Amer. J. Clin. Path., Tech. Suppl.*, 4, 65–8. 1940.

A trichrome stain for astrocytes is described which gives results as good as those obtained with gold or silver impregnation and is suitable for bulk staining. It is better than Masson's trichrome stains for color photomicrography using Kodachrome or color separation. The method is as follows: Fix in formalin. Double embed (Beyer: Amer. J. Clin. Path., Tech. Suppl., 2, 173, 1938). Cut sections 5 μ. Remove paraffin. Run thru alcohols to water. Apply Muller's mordant, 3–12 hr. Wash in 3 changes of tap water and 1 of dist. water. Apply Hansen's hematoxylin, 5 min. or longer (Soln. A: ferric alum, amethyst cryst., 10 g.; ammonium sulfate, 1.4 g.; dist. water, 150 cc., heated below boiling until dissolved, and cooled. Soln. B: hematoxylin, 1.6 g.; dist. water, 75 cc., dissolved by heat, and cooled. A is added to B gradually with constant stirring, and when deep violet, as seen on filter paper, is heated slowly to boiling and boiled 30 sec., then cooled in ice water.) Wash in running tap water, 2 min. Differentiate in 2% acid alcohol, 15 sec. Wash in tap water. Rinse in ammonia water (1–2 drops to 100 cc.). Wash in dist. water. Stain in ponceau-fuchsin, 5 min. (Soln. A: ponceau de xylidine, either Eimer and Amend, Krall or Grübler, 1 g.; glacial acetic acid, 1 cc.; dist. water, 100 cc. Soln. B: acid fuchsin, Commission cert., 1 g.; glacial acetic acid, 1 cc.; dist. water, 100 cc. To 90 cc. of 0.2% acetic acid 6.6 cc. of soln. A and 3.3 cc. of soln. B are added). Wash in 2 changes of tap water and 1 of dist. water. Stain 5 min. in: phosphotungstic acid, 3 g.; orange G (Hollborn), 2 g.; dist. water, 100 cc. Mix in a mortar. Rinse quickly in 1 change of dist. water. Put in 0.5% Wasserblau (Grübler, sold by Pfaltz and Bauer) 10 to 15 min. Rinse in dist. water containing 1% of the Wasserblau staining solution. Run thru 2 changes of 95% alcohol, 2 changes of abs. alcohol, and then xylol. Mount in neutral balsam or gum damar.—*G. H. Chapman.*

CIARDI-DUPRÉ, G. **Accorgimenti per allestire economicamente serie istologiche.** *Monit. Zool. Ital.*, 50, 314–5. 1940.

An inexpensive substitute for slides and cover slips to mount large sections of embryonal and fetal tissue is described. Sections are mounted on old photographic glass which has been cut to the proper size and from which the gelatin has been peeled. Sheet cellophane, weighing 140–160 g. per sheet measuring 1×1.50 m. is used to cover the sections. Damar balsam is recommended as a mounting medium, and the cellophane, cut to appropriate size, is applied as one would a cover slip. It is important that the cellophane be under pressure for at least 24 hr. so as to obtain a level surface. The author suggests pressing between two thick pieces of cardboard held by a pair of elastic bands.—*A. B. Dawson.*

KARR, J. W. **A substitute for balsam and damar.** *Amer. J. Clin. Path., Tech. Suppl.*, 4, 70. 1940.

Nevillite (Neville Company, Neville Island, Pittsburgh, Pa.) is suggested as a mounting medium.—*George H. Chapman.*

MC NAMARA, W. L., MURPHY, BERTA, and GORE, W. A. **Method of simultaneous fixation and decalcification of bone.** *J. Lab. & Clin. Med.*, 25, 874–5. 1940.

The following method of simultaneous fixation and decalcification can be used for the majority of special staining procedures: Cut sections of bone not more than 8–10 mm. thick with a scroll saw. Place them in the decalcifying solution at 37° C. Dissolve 10 g. of $HgCl_2$ in 300 cc. of dist. water with heat and cool it. Dissolve 30 g. of trichloracetic acid in 100 cc. of water and add 5 cc. HNO_3, 40 cc. formalin and 50 cc. 95% alcohol. (Mix the two solutions.) Change this solution daily until tissue is soft. Keep in running water for 24 hr. (or in 2% NH_4OH for 12 hr. and running water for 24 hr.) Place in 80% alcohol, 2 hr.; 95% alcohol, 6 hr.; abs. alcohol, 16–24 hr.; xylol, 5–10 min.; cedar oil, 24 hr.; xylol (2 changes), 30 min.; 56°–58° paraffin (3 changes), 4–6 hr.; and embed. The average bone decalcifies in 3–5 days. If longer than 7 days is required, nuclear staining is impaired. By this technic tissue distortion is minimal and the staining capacity of the nuclei is undiminished.—*John T. Myers.*

O'BRIEN, H. C., and HANCE, R. T. **A plastic coverglass, isobutyl methacrylate.** *Science*, 91, 412. 1940.

Natural resins, like Canada balsam and gum damar, harden slowly and turn yellow in time. Nevillite, or Clarite, recently suggested by the General Biological Co., dries rapidly and is practically colorless. Isobutyl methacrylate, a plastic made by the du Pont Co. and costing about $1.00 a lb., is similarly found to dissolve in benzene and xylene, giving a colorless solution. Moreover, it dries very hard within 5–10 min. and yields a preparation somewhat more brilliant than those mounted in Clarite. Its refraction index is 1.477, very near that of glass. Isobutyl methacrylate may also be used to replace the coverglass by simply dipping the slide into a thin solution of the plastic and draining off the excess. Immersion oil does not affect its surface, which can be wiped clean with alcohol and soft paper. The film produced is very thin, thus interference with light transmission is very small; but it will scratch. Scratches are removed by dipping the slide into the same plastic solution. Lastly, the film obtained can be written on with ordinary ink for purposes of labeling.—*J. A. de Tomasi.*

WARD, MARGARET C. **Gum damar in place of thin celloidin for frozen sections.** *Amer. J. Clin. Path., Tech. Suppl.*, 4, 71. 1940.

Gum damar, used to affix frozen sections to slides, does not interfere with staining. The method described is as follows: Coat the slide with gum damar. Float section onto the slide and drain. Flush with 70% alcohol and blot with a blotter moistened with 70% alcohol. Stain as usual. Keep the slide horizontal while in xylol because it tends to loosen the section.—*G. H. Chapman.*

DYES AND THEIR BIOLOGICAL USES

FIGGE, F. H. J. **Squid melanin: A naturally occurring reversibly oxidizable pigment.** *Proc. Soc. Exp. Biol. & Med.*, 44, 293–4. 1940.

The ink of the squid (*Loligo pealii*) is a highly concentrated suspension of melanin, the particles of which are visible only by dark field illumination. Comparisons between this melanin and synthetic dopa melanin indicate marked reduction with sodium hydrosulphite and re-oxidation with potassium ferricyanide in both instances, whereas natural melanin exhibits more sluggish reactions. Squid melanin contains some substance absorbing about 10% of the light which is not reversibly oxidizable.—*M. S. Marshall.*

ORR, J. W. **The histology of the rat's liver during the course of carcinogenesis by butter-yellow (*p*-dimethylaminoazobenzene).** *J. Path. & Bact.*, 50, 393–408. 1940.

The dye "butter-yellow", obtained from British Drug Houses, Ltd. under the name "dimethyl yellow", was dissolved in oil and then added to the diet for the induction of visceral neoplasms.—*S. H. Hutner.*

ANIMAL MICROTECHNIC

BRUNSCHWIG, A., SCHMITZ, R. L., and JENNINGS, S. **Selective localization of Evans blue (T1824) in subplacental portions of entoderm in the rat.** *Proc. Soc. Exp. Biol. & Med.*, 44, 64–6. 1940.

The injection of pregnant white rats with 4 mg. of trypan blue was followed by localization as small blue granules in the columnar cells of the subplacental portions of the visceral entoderm. The experimental check followed observations of the localization of Evans blue (T1824), which accumulated in the macrophages and fibroblasts of the stroma around malignant but not benign tumors, following the injection accidentally of a pregnant rat.—*M. S. Marshall.*

DIGGS, L. W. and PETTIT, V. D. **A comparison of methods used in the detection of the sickle-cell trait.** *J. Lab. & Clin. Med.*, 25, 1106–11. 1940.

Five methods of detecting sickle cells were compared. The best one is the following moist stasis technic of Scriver and Waugh (Canad. Med. A. J., 23, 375, 1930): Place a rubber band around the proximal third of the finger and allow it to remain for 5 min. Puncture the distal end of the finger, seal a drop of the dark blood under a cover slip with vaseline and examine microscopically.—*John T. Myers.*

GOMORI, G. **A method for staining of carious lesions in teeth.** *Proc. Soc. Exp. Biol. & Med.*, 44, 250–3. 1940.

The following method permits rapid and accurate work, with human teeth or with large numbers of rat jaws: Fix in 80–95% alcohol or neutral formalin and wash in repeated changes of dist. water. Impregnate with 0.25–0.5% $AgNO_3$ for 12–24 hr. and wash for at least 24 hr. Reduce in 5% $Na_2H_2PO_2$ for 24 hr. and wash several hours in tap water. Fix in 2% $Na_2S_2O_3$ ("hypo") for 12 hr. and wash under the tap several hours. Stained jaws may be dehydrated in alcohol, cleared in cedar oil and examined directly under the dissecting microscope or they may be decalcified in 5–10% sulfosalicylic acid (not mineral acids) and embedded in celloidin. Carious areas are black; healthy areas are unstained.—*M. S. Marshall.*

IGNESTI, UGO. **Avvertenze per la colorazione con la fucsina di Ziehl di materiale fissato in liquidi contenenti acidi.** *Monit. Zool. Ital.*, 51, 80–2. 1940.

A modification of the Vannucci-Mallory method is proposed in which the acid fuchsin is replaced by Ziehl's fuchsin according to the method of Gallego. This modification permits differential staining following fixation with alcohol or fluids containing acid (Susa, Gilson-Carazzi, Zenker acetic, picro-sublimate, Sanfelice, and Bouin).

The essential modification is the omission of acetic acid from the solutions used in steps 1 and 3 of the following procedure: 1) Stain for 3–5 min. in dil. carbol fuchsin (5 g. Ziehl's fuchsin, dissolved in 10 cc. dist. water). 2) Wash in dist. water. 3) Differentiate for 5 min. in dil. formalin (formalin, 3 g., dissolved in dist. water, 10 cc.). For the rest of the steps, the original method is followed.

If nuclear staining is too intense, the solution in step 1 may be made with 2 g. Ziehl's fuchsin, and the time of staining reduced to 1–3 min.—*A. B. Dawson.*

JÄGER, R., and JÄGER, F. **Fluoreszenmikroskopie im auffallenden Licht unter besonderer Berücksichtigung der Struktur der Oberfläche der lebenden Haut und der Vereinfachung der Hilfsmittel.** *Zts. wiss. Mikr.*, 56, 273. 1939.

This is a technical description of various methods employed in obtaining sharp surface contour pictures of the epidermis. The latter is rendered sensitive to ultra-violet light by washing the parts to be examined with a dil. solution of some flurochrome such as Primulin "O" (Grübler) or Auramine (Hollborn). The usual opaque illuminators are used.

The method should find various practical applications in studying the effects of soaps and cosmetics, industial hazards, etc., upon the epidermis.—*J. M. Thuringer.*

JUGE, JEAN. **Les potentialities morphogenetiques des segments du membre dans la regeneration du Triton** (Autopode). *Rev. Suisse de Zool.*, 47, 65–133. 1940.

A method for contrast staining of bone and cartilage in cleared specimens is described as follows: Fix 24–48 hr. in 4% formalin. Wash in running water several hours. Transfer to 70% alcohol for 24 hr. Remove the skin. Stain 1–3 hr. in acetic methyl green (methyl green, 3 g.; 70% alcohol, 100 cc.; acetic acid, 2 drops). Differentiate in several changes of 70% alcohol several hours until the desired color is reached; 95% alcohol, 2 hr.; abs. alcohol, 2 hr. Stain 12–24 hr. in alizarin (saturated solution of alizarin in abs. alcohol, 1 cc.; abs. alcohol, 100 cc.; acetic acid, 5 drops). Transfer to abs. alcohol for 2 hr. Clear in toluol or benzol for ½ hr.

Material may be preserved in a mixture of 27 cc. methyl salicylate with 7 cc. isosaphrol, or in methyl salicylate alone. Cartilage appears green (but loses its color at the end of several months); bone is red.—*A. B. Dawson.*

KAUFMANN, W. **Occurrence of special cell groups at vascular pole of glomerulus in mammalian kidneys.** *Proc. Soc. Exp. Biol. & Med.*, 44, 227–30. 1940.

This is a report of a study of the juxtaglomerular corpuscles of Goormaghtigh, composed of agglomerations of cells. The author recommends fixation in Bouin's or Zenker's solution while fresh, paraffin embedding and sectioning serially, 4–6 μ. Hematoxylin-eosin fails to give adequate detail, Masson's trichrome stain is better and Mallory's phosphotungstic-acid-hematoxylin reveals good nuclear detail. Cytoplasmic vacuolization is visible by this method or with Mallory's anilin blue connective tissue stain. Intercellular fibrils are well brought out with Masson's stain or better with silver stains.—*M. S. Marshall.*

LANDAU, E. **Quelques réflexions sur les procédés d'imprégnation des neurofibrilles par le nitrate d'argent.** *Bull. d'Histol. Appl.*, 17, 65–80. 1940.

A method is described for the silver impregnation of paraffin sections from the nerve fibers of the central nervous system, as follows: Use material that has been fixed in formalin. Fasten the sections to slides with a very weak solution of gelatin, heating to an optimum temperature of 35° C. Remove paraffin and place in a 10% solution of neutral formalin 12–24 hr. or longer, protected from light. (The authors do not consider treatment with 95% alcohol desirable before impregnation.) After removal from formalin, rinse in dist. water, and transfer, in the dark, to a 20% solution of $AgNO_3$. Hold in an oven at 35–40° C. for 1–2 hr. according to the thickness of the section. Rinse 1 sec. in dist. water and transfer to 20% ammoniacal silver. After 5 min. rinse briefly and reduce the silver by several drops of 1% neutral formalin. Repeat at intervals, controlling the reduction of the silver under the microsope.

The impregnation is continued until the desired result is obtained. The reduction may then be stopped by formalin, by a thoro washing in dist. water, or by the method proposed by Gros. After toning in $AuCl_3$ and fixing in $Na_2S_2O_3$, the sections may be differentiated in a ½% aq. solution of potassium ferricyanide or 10–15% KI. When the differentiation is finished, the section is washed several hours in running water, dehydrated, and mounted either in Canada balsam or in the following fluid preferred by the authors: dist. water, 30 cc.; gum arabic, 30.0 cc.; refined sugar, 30 g.; glucose, 5 g.

This method has been used successfully for the cerebral cortex, the cerebellum and the bulb.—*Jean E. Conn.*

MC CARTER, JOHN C. **A silver carbonate method for oligodendrocytes and microglia for routine use.** *Amer. J. Path.*, 16, 233–5. 1940.

The following silver method is recommended for tissue of the central nervous system: Fix in 10% formalin. Treat frozen sections (20–25 μ) with a 1% solution of strong ammonia in dist. water for a few minutes, if the material is recently fixed, or over night if fixed for several weeks. Without washing, transfer to 4% aq. HBr (10% soln. of conc. hydrobromic acid) at 37° C. for 1 hr., wash in 2 changes dist. water and put into 5% aq. Na_2CO_3. Add an equal volume of 5%

aq. ammonium alum (disregard ppt.) and leave the sections in this mixture for 1 hr. to 2–3 days. Wash in 2 changes dist. water and stain in Hortega's strong silver carbonate solution (5 cc. 10% $AgNO_3$, plus 20 cc. 5% Na_2CO_3, plus strong ammonia water to just dissolve ppt., filter and add 20 cc. dist. water) for 2–5 min. Reduce, without rinsing, in 1% formalin and agitate by blowing on the sections. Wash well in dist. water, gold-tone and fix in hypo in the usual manner. Dehydrate the sections after putting on the slide and cover in balsam. The method combines modifications of Hortega's method for oligodendrocytes and microglia with those of Globus and Penfield and adds the ammonium alum mordant; the time the sections remain in the first ammonia bath is varied to suit the time of fixation.—*H. A. Davenport.*

REXED, B., and WOHLFART, G. **Über Färbung mit gepufferten Säurefuchsinlösung.** *Zts. wiss. Mikr.*, **56**, 212–5. 1939.

The authors attribute the extreme variability of the action of acid fuchsin as used in Mallory's connective tissue stain to the varying pH of its solution. The reaction of a freshly prepared 0.1% solution of acid fuchsin is pH 4.49, and any alkalinity raises the pH sufficiently to render the stain inert. The following recommended formula gives a solution of pH 3.29 ± 0.01: acid fuchsin, 1.0 g.; N/10 HCl, 60.0 ml.; dist. water, 900 ml.; Sörensen's citrate solution, 40 ml. (citric acid crystals 21.0 g., N/1 NaOH 200 ml., dist. water to make 1 L.).

Practically all organs stain in range of pH 3–4. The red blood cells alone stain at pH 5–7.—*J. M. Thuringer.*

RÖHLINGER, H., and REITZ, M. **Die Methode der Schnittveraschung und ihre Bedeutung für die physikalisch-therapeutische Forschung.** *Zts. wiss. Mikr.*, **56**, 361. 1939.

The importance of the spodogram in dermatology for the control of physical therapy measures is discussed. Every functional change of the skin is contingent upon a shifting of its electrolytic components.

The presence of calcium, magnesium, phosphates, iron, and silicic acid are determined as follows: Fix the living tissue in abs. alcohol 48–72 hr. and imbed in paraffin. Cut sections 10 μ, mount with dist. water and incinerate with aid of microincinerator. Postmortem material is unsuitable because of its altered cell permeability, diffusion phenomena, and change in pH. The preparations are unstable and photomicrographs must be made immediately.

To determine total Ca salts: breathe gently on the spodogram to convert the water soluble Ca salts into carbonates which are practically insoluble. The residue may be washed from the preparation with dist. water.

The Mg pictures obtained after previous removal of Ca salts are less definite than when made from the fresh spodogram. It is demonstrated by treating the spodogram for 3 min. with a 1% tetraoxyanthrachinon solution and removing the remainder with a 10% solution of antipyrin. Lastly, the sections are treated for 30–40 min. with dist. water (40–50° C.) using several changes.

The demonstration of phosphates is more difficult because of precipitate formation on the spodogram; however, they may be shown in the total ash as well as Ca picture. To overcome this difficulty a drop of abs. alcohol may be placed carefully on the spodogram and cover slip. The reagent ferric acetate (concentration not mentioned) is dropped at the side of the cover slip and drawn thru by holding a bit of filter paper to the opposite side. A few drops of alcohol mixed with the reagents hastens this process. This procedure should be repeated 3 or 4 times. A solution of 1% acetic acid in 33% alcohol is similarly drawn thru. Ferric phosphates are formed while other salts are removed by acetic acid. The iron salts are rendered visible by drawing thru first 2–3 drops of a potassium ferrocyanide solution followed by 4–5 drops of 1% HCl.

Iron may be demonstrated with the Berlin blue or still better the Turnbull reaction, as follows: precipitate with ammonium sulphide, add 20% potassium ferrocyanide in the presence of HCl and observe the blue color reaction.

Silicic acid is isolated by treating the spodogram ½–1 min. with 1–2% aq. HCl. The remainder of the spodogram consists of silicic acid since all other salts are dissolved and washed away by HCl.—*J. M. Thuringer.*

ROSKIN, G., and KIRPICHNIKOVA, E. **Coloration vitale par ionophorèse.** *Bull. d'Histol. Appl.*, **17**, 146–7. 1940.

A method is described for vital staining of animal cells by means of ionization. Colored ions are introduced into the living organism by means of a continuous current from a storage battery having an intensity of 0.25 milliamperes for an electrode of 1 sq. cm. Tin plates are used for electrodes with the active one a little smaller than the other. Various dyes can be used; e.g., methylene blue which has a colored positive ion. The electrodes are attached to the animal after the hair has been removed. A pad of gauze soaked in a 0.5–1.0% solution of methylene blue is placed under the active electrode, and gauze wet with water is placed under the other electrode. The current is allowed to pass for 20–30 min.

Coloration can be observed after the first electrification, but it becomes more intense after this has been repeated on several successive days. With white mice coloring has been observed, not only in the cells of the skin, but also in the outer layers of the muscles. After killing the animal, the tissues are fixed in the following solution: Sat. aq. picric acid, 6 parts; sat. aq. ammonium molybdate, 2 parts; formalin, 2 parts. They are then passed thru alcohol and embedded in paraffin.

This method can also be used for vital staining of nerves.—*Jean E. Conn.*

SCHORR, E. **A new technic for staining vaginal smears.** *Science*, **91**, 321. 1940.

The conventional hematoxylin-eosin-water-blue technic of Papanicolaou for vaginal smears is not entirely satisfactory when stress is put on the detection of cornification, a cytoplasmic change taken as a measure of ovarian function. The following modification of the Masson trichrome stain has, however, proved useful, because the sequence of contrasting colors produced is comparable to a chemical titration of the cell charge of the smear. Fix (no time given) the wet smear in 95% alcohol-ether, 1:1; run down to water. Stain 2 min. in Harris' hematoxylin; rinse 4–5 times in water, and let stand 5 min. in running water. Stain 5 min. in ponceau-acid-fuchsin-orange-G; rinse 3–4 times in water. Mordant 10 min. in 3% phosphotungstic acid; rinse 3–4 times in water. Counterstain 8 min. in light green; do not wash. Acidify 3 min. in 0.25% acetic acid; do not wash. Dehydrate, clear, and mount. For the composition of the trichrome stain reference is made to Foot's description (Amer. J. Path., **14**, 245, 1938).—*J. A. de Tomasi.*

TAFT, A. E. **A supplementary method for the study of Arachnopia.** *Science*, **91**, 272. 1940.

The usual preparations of brain and cord tissue give an inadequate idea of structure and histological relations of leptomeninges. Such stained preparations can, however, be supplemented with others which, prepared by the following simple technic, will often yield further valuable information: Float fragments of fresh or formalin-fixed tissue from water onto a glass slide, add a drop of glycerin, and put cover in place, with the application of some pressure. The material is thus made suitable for dark field work, a technic especially valuable when the study of vascular arrangement and meningeal concretions is called for.—*J. A. de Tomasi.*

TAKEYA-SIKO. **Markscheidenfärbemethode am Gefrierschnitt.** *Zts. gesam. Neurol. u. Psychiat.*, **169**, 216–9. 1940.

As a supplement to Schroeder's article (abs. Stain Techn., **15**, 37) two methods for myelin sheaths are presented. The first is the Sugamo method as follows: Fix in 10% formalin; wash in running water 12–36 hr. Cut frozen sections (about 30 μ). Place 3–5 min. in 50% alcohol (agitate well); transfer to dist. water, then mordant 6–15 hr. at 37° C. in $K_2Cr_2O_7$, 5 g.; chromalum, 2 g.; dist. water, 100 cc. with sections flat and submerged in the fluid. Rinse 2–3 times in dist. water and stain 2–6 hr. at 37° C. in Kultschitsky's acetic hematoxylin. Transfer to water (use perforated spatula as sections are brittle); wash thoroly; then differentiate in 0.3–1.0% $KMnO_4$ 20–30 sec., one section at a time. Rinse in dist.

water and treat with a fresh mixture of equal parts of 1% oxalic acid and 1% Na_2SO_3. Wash thoroly in tap water; dehydrate; clear in creosote-xylene and mount in balsam. The Yatusiro method is like the first with the following exceptions: Omit the mordant; stain in aged Delafield's alum-hematoxylin 12–24 hr.; differentiate in borax 2 g., potassium ferricyanide 2.5 g., water 100 cc.—*H. A. Davenport.*

WOLF-HEIDEGGER, G. **Die Anwendung von Kava-Kava bei der Fixierung des Dünndarms und anderer Hohlorgane.** *Zts. wiss. Mikr.*, 56, 417. 1940.

Postmortem contractions of smooth muscle in the alimentary tract of mammals produce distorted histological pictures. To overcome this difficulty an infusion of powdered Kava-Kava root (*Piper latifolium*) is recommended.

The procedure is as follows: Heat 250 ml. Ringer's solution slowly to 80° C.; add 15 g. Kava-Kava powder, agitating constantly. When cooled to 37° C., take a few ml. of this solution and add a small amount of Merck's diastase, mix thoroly and add to the remainder of the Ringer's. Let digest in incubator for 2½ hr., filter, discard ppt., and proceed to inject animal. Any of the usual fixatives may follow this treatment.

The results were excellent on cats while rabbits receiving the same preliminary treatment did not respond to the Kava-Kava relaxation which may be attributed to their being vegetarians.—*J. M. Thuringer.*

PLANT MICROTECHNIC

GORDON, W. E. **A labor-saving technique for leaf samples in histological work.** *Science*, **91**, 390. 1940.

For the purpose of recording the source of each leaf sample and maintaining its identity thruout the various manipulations in the laboratory, the following procedure is recommended: By means of a crow quill pen, put duplicate numbers in India ink on fresh leaves in the region from which the sample is to be taken. Punch out a disk-like portion which includes one of these numbers, and use this as a sample to embed and store for future sectioning. The other number is left to record the source and location of the sample taken; the leaf bearing it is pressed and dried for filing. As many as 15 such samples from the same specimen can be preserved, fixed, dehydrated and embedded at the same time with the sample bearing the number at the bottom of the block, so that it is legible thru the thin layer of paraffin. The numbers in ink are not washed off by chrom-acetic, or formol-acetic-alcohol fixatives, nor by the common alcohols, dioxan, or chloral hydrate.—*J. A. de Tomasi.*

HILLARY, B. B. **Use of the Feulgen reaction in cytology. I. Effect of fixatives on the reaction.** *Botan. Gazette*, **101**, 276–300. 1939.

"In vitro" experiments with nucleic acid impregnated agar blocks using four different types of fixatives showed that the Feulgen nucleal stain gives two types of staining curves, depending on the presence or absence of chromic acid in the fixative. With fixatives not containing chromic acid, the maximum stain is produced by hydrolysis at 60° C. extending from 4 to 8 min.; after that time a gradual falling off of the stain takes place, till at 30 min. no stain is visible. With fixatives containing chromic acid, the maximum stain is produced by hydrolysis at 60° C. for from 5–30 min. That this greater retention of the stain with increased hydrolysis is due to chromic acid was established by testing the individual ingredients of the fixatives separately. Most of the ingredients of the fixatives, if they are present at the concentration normally used, do not interfere with the stain. Tannins, which occur widely in plant cells and which have been reported to inhibit the stain, were found to have no inhibitory effect unless a fixative was used that contained formalin. The nuclei of various plants from different divisions thruout the plant kingdom when treated in the same manner as the agar blocks showed the same type of staining curves. Causes of the previously reported negative stain reactions in many of these plants are discussed.—*B. B. Hillary.*

THOMAS, J. WARRICK. **A comparison of cedar oil and other materials in the making of slides of atmospheric pollen.** *J. Lab. & Clin. Med.*, 25, 1086–90. 1940.

Comparative studies were made of atmospheric pollen slides prepared with a glycerin jelly, glycerin vaseline, corn oil, almond oil and cedar oil. Glycerin jelly, containing a trace of methyl green (proportion not given) is desirable where structural detail is desired, but not for routine use. Cedar is preferable to other oils because it remains in the slide area to which it is applied, is more adhesive and makes the pollen grains more translucent. Cedar oil preparations may be filed either mounted with Canada balsam and cover slip, or unmounted in dust proof slide boxes.—*John T. Myers.*

MICROÖRGANISMS

HORVATH, J.v. **Die Bedeutung des Zuchtwassers und der Fixierung in der Mikrotechnik der Protozoen. I. Die Wirkung des Eisennitrates bei Anwendung von Anilinfarben.** *Zts. wiss. Mikr.*, **56**, 291. 1939.

The staining of protozoa can be influenced by adding 5 drops of 0.13% $Fe_2(NO_3)_3$ to 10 ml. of the hay infusion or other culture medium in which they are growing. The action of the iron salt is said to be entirely unlike that of a mordant since it has no effect after fixation. It may be used in relatively high concentrations "in vivo" without affecting the viability of the organisms, and may be followed with one of the usual fixing fluids suitable for protozoa. The specifity of the various stains may be altered by regulation of the amount of $Fe_2(NO_3)_3$ added.—*J. M. Thuringer.*

LEVADITI, C., REINIÉ, L., STAMATIN, LE-VAN-SEN, and BEQUIGNON, R. **Ultravirus et fluorescence. Le virus vaccinal.** *Ann. Inst. Pasteur,* **64**, 359–414. 1940.

The following modified Hagemann technic is offered for the use of fluorescent dyes in the microscopy of viruses: Smear thick suspensions of elementary bodies on a slide, dry 1 hr. at 37° C. Agitate slides in dist. water for 10 min., dry 1 hr. at 37° C. Stain 5 min., rinse in dist. water, examine dry preparations by fluorescence. The authors used the Zeiss cardioid dark-field apparatus supplied with quartz stage accessories to enable use of an ultraviolet light source. The best dye, thioflavine, and the less suitable dyes, primuline, thioflavine S, auramine O, rhodamine B, trypaflavine, uranine A and eosin G, were obtained from Hoerlein. The elementary bodies are brilliantly fluorescent against the dark background. Silver impregnation staining methods afford a good check on fluorescence methods.—*S. H. Hutner.*

HISTOCHEMISTRY

CRÄMER, G. **Ein Verfahren, Nitrate im Gewebe sichtbar zu machen.** *Zentbl. allg. Path.*, **74**, 241–4. 1940.

The reagent, diphenyl-endo-anilo-dihydro-triazole (called "Nitron" by Busch), forms insoluble salts with nitrates and these can be demonstrated in tissue by examination with polarized light. The method is as follows: Dissolve 10 g. "Nitron" with 100 cc. of 5% acetic acid, heat (temp. not given). Cut fresh (unfixed) tissue with a freezing microtome (cool knife) and receive the section on a slide. Put 1–2 drops of hot "Nitron" solution on a cover glass and cover the section with it. Store ½ hr. in a refrigerator to assist crystallization of the nitrate. Examination with polarized light reveals the presence of nitrates as doubly refractive zones. Methylene blue can be added to the "Nitron" reagent if desired. Sections must be examined immediately after removal from the refrigerator. A discussion of interfering substances is given. Further information: Busch, M., Ber. deut. chem. Ges., **38**, 861.—*H. A. Davenport.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the July number of this Journal.

STAINS CERTIFIED JUNE 1, TO AUG. 31, 1940*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Phloxine	CPh-3	90%	For histological staining	June 17, 1940
Wright stain	CWr-13	....	As blood stain	June 27, 1940
Rose Bengal	CRb-3	80%	As bacteriological stain	June 28, 1940
Brilliant cresyl blue	NV-19	55%	For vital staining of blood	July 8, 1940
Methyl orange	LM-1	91%	As histological counterstain	July 16, 1940
Methyl orange	NM-6	91%	As histological counterstain	July 16, 1940
Pyronin Y	NP-8	52%	As constituent of Pappenheim stain	July 22, 1940
Methylene blue thiocyanate tablets	NAt-2	1.31–1.65%	For use in reduction test of milk	July 23, 1940
Hematoxylin	FH-16	....	As histological and cytological stain	Aug. 2, 1940
Fast green CF	LGf-1	93%	As histological and cytological counterstain	Aug. 10, 1940
Malachite green	NMg-8	93%	As histological and bacteriological stain	Aug. 12, 1940
Giemsa stain	NGe-6	....	As blood stain	Aug. 21, 1940
Tetrachrome stain	NMn-11	....	As blood stain	Aug. 31, 1940

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

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